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Black sheep slink home from Geneva

On the principle that one man's victory is another's defeat, last week's proceedings at Geneva of the second review conference of the Non-Proliferation Treaty (NPT) were not a simple failure. The nuclear powers, it is true, are understandably crestfallen at having failed to persuade their fellow signatories that they have done their best to keep their side of the bargain, especially in strategic disarmament and assistance with civil nuclear technology. But many among the non-nuclear power signatories are correspondingly cock-a-hoop at having stuck to their subversive guns during the Geneva meeting, insisting that the record of the nuclear powers in the past five years does not justify a clean end-of-term report. The non-nuclear powers insisted at Geneva that, if the nuclear powers really wanted a consensus declaration, they would have to agree on the spot to behave as if the Salt II treaty had been ratified, and embark on multilateral negotiations on tactical nuclear weapons and other steps judged unacceptable (see *Nature* 11 September). The failure to reach agreement is also, however, a failure in a broader sense. The signatories of the Non-Proliferation Treaty are loosely held together by a variety of pressures. Some have a genuine conviction that the NPT is a valuable instrument of arms control. Others have been persuaded to sign through coercion by nuclear powers. Others hope that they will have an inside track in nuclear assistance. The most immediate danger now is that this conglomerate of signatories will begin to fall apart. Only a few defections from the treaty (which includes a provision for giving three months' notice) could do a great deal of damage. How likely is such a course of events? What would be the consequences? And what now should the nuclear powers do to make up for the disappointment of Geneva?

Whatever the fall-out, rapid defection is unlikely. Indeed, even the most disaffected signatories will probably stay in the club, at least for the time being. The opportunity to tweak the lions' tails that Geneva provided may after all recur. Political pressures will also persist, as will the tangible threat that withdrawal from the treaty, and from the system of safeguards operated by the IAEA at Vienna, would deprive many states of much-needed nuclear supplies. And a great many signatories will know that they may jeopardize the whole non-proliferation system if they now rock the boat by walking out in a huff. Defections will come, if at all, only much later and for different reasons. No government now a signatory of the NPT is going to embark on the manufacture of nuclear weapons lightly, and without a careful assessment of the political as well as strategic risks of such a step. For most countries interested in such calculations, the political risks of resigning from the NPT are likely to be much smaller than the other risks. Accordingly the failure of the review conference to agree on a final statement cannot be decisive in any present signatory's calculations of the future. The consequences are most likely to be that governments which have so far held out against signing the NPT will now be even more reluctant. In short, the setback at Geneva last week does not, of itself, imply the end of the NPT system as it is.

What the ructions at Geneva do imply, however, is that the non-nuclear powers are much more fierce in their discontent with the past five years' progress in arms control than the nuclear powers had expected. To be sure, the nuclear powers (especially the superpowers) went to Geneva expecting trouble. They expected to be hauled over the coals, told that their performance (and especially their failure to ratify Salt II) was inexcusable and warned that they would have to do much better in the future. In the

event, they were not let off the hook as easily as they had expected. Indeed, they were not let off the hook at all. The demand that the signatories of Salt II should behave as if they had ratified it was plainly unacceptable to the Soviet Union. (Although the United States government has often said it would behave as if Salt II had been ratified, President Carter's representatives at Geneva would have been acutely aware that a President Reagan might think differently.) Both superpowers would have shrunk from the demand that Salt III (which is supposed to be concerned with tactical weapons) should be thrown into the Committee on Disarmament, and perhaps with reason.

In a quite dramatic way, however, the NPT review conference has been a psychological shock to the nuclear powers and indirectly to the non-nuclear members of NATO and the Warsaw Pact. Hitherto, it has been possible for the nuclear powers to excuse the slow pace of their arms control negotiations on a variety of grounds — trouble in Afghanistan, uncertainty in the Middle East, presidential elections here or there. What the non-nuclear powers told them at Geneva is that they must make progress whatever the circumstances. This will be a hard prescription to follow. They are, for example, being required seriously to contemplate the completion of the Comprehensive Test-Ban Treaty negotiations and then of giving up all nuclear tests for good (see *Nature* 14 August). The prescription is all the more authoritative because the dissidents at Geneva were not just disaffected developing countries — Sweden and (less openly) countries such as the Netherlands had unkind things to say about the nuclear powers.

How, then, will the black sheep respond? It is tempting to think that Mr Edmund Muskie's statement last week that the US government is now prepared to talk to the Soviet Union about nuclear missiles in Europe is one reaction to what happened at Geneva. Certainly that is a step forward, and one that will bring some good cheer in West Germany as well as elsewhere in Western Europe. But the connection is unfortunately too indirect. There is very little evidence that the Secretary of State has yet had time to consult other interested European states about the form negotiations of this kind should take — yet the European members of NATO are anxious that the next Salt treaty should not be an exclusively Soviet-American deal. Mr Muskie also studiously avoided mentioning the Committee on Disarmament in this context. And he (like everybody else) knows that he will not be able to deliver on his promise if his president is not re-elected. Similarly, the ratification of Salt II must be held up at least until the new Senate assembles in the new year, and may then be just as unlikely as it has been for the past year.

If the nuclear powers seek, as they should, some way of disarming the criticisms they encountered at Geneva on arms control, they have very little choice but to push ahead with the Comprehensive Test-Ban Treaty. The report on these negotiations put out at the end of July was a surprisingly cheerful document, suggesting that much of the technical work has now been done. A little more talking might be enough to do the trick. Indeed, for the past few weeks some of those concerned with the negotiations have been saying that the only missing ingredient in the Comprehensive Test-Ban Treaty is the will of the superpowers to sign on the dotted line. Although plainly an exaggeration (for the Soviet demand that there should be as many remote seismic monitoring sites in the United Kingdom as in the United States remains unresolved), this must be a good approximation to the truth. The question now is whether the drubbing at Geneva will



persuade the superpowers to complete the treaty.

The chances are slim. The political inhibitions of the past few months persist. In the circumstances, the nuclear powers should think of taking more seriously the persistent demand of the non-aligned signatories at Geneva — that more of the process of arms control should be entrusted to the Committee on Disarmament in Geneva. It is entirely understandable that the committee has earned itself a bad reputation. For the first decade or so it was a talking shop for the rehearsal of idealistic but also unrealistic schemes for disarmament. Pilloried at regular intervals, the superpowers took care to avoid trouble (and to nullify the committee's work) by the device of their control of the chairmanship of the committee. Since the reorganization (and enlargement) of the committee five years ago, however, its proceedings have been more temperate and more valuable. Moreover, the committee has the virtue of being the only forum of its kind in which both France and China are represented. Because at some stage neither the existing Partial Test-Ban Treaty nor the putative Comprehensive Test-Ban Treaty can survive indefinitely if France and China stay outside, the nuclear powers have everything to gain if questions of French and Chinese adherence to these schemes are seriously taken up. What is needed is a definition of the circumstances in which France and China would sign. The old device of cutting off other people's military production of nuclear explosives is unlikely now to be sufficient. The Geneva black sheep might at least find they were not the only animals of that colour if they used the Committee on Disarmament as a means of exploring such questions.

The nuclear powers have not merely to meet criticisms that they have dragged their feet on strategic arms control (Article VI of the NPT) but also that they have fallen short of their obligations to help with the spread of civil nuclear technology. Here, by good luck, they have an early opportunity to disarm their critics. At the end of this month, the IAEA Committee on Assurance of Supply will meet for the first time in Vienna, chiefly to discover which members of the IAEA have chosen to belong (it is a free-for-all) and to decide when to meet next. Potentially, however, the committee is a means by which the nuclear suppliers (not all of them nuclear powers) could help meet the just criticisms that have arisen of their supply practices. As things are, many governments fear that, after building a nuclear reactor, they may be unable to buy fuel for it on the world market. Only last week, the US Congress finally overrode President Carter's wish to send a consignment of enriched uranium to India; the uranium is contracted for on both sides, but prohibited by the Anti-Proliferation Act of 1968. South Africa is in similar difficulties. Nobody would quarrel with the objectives of this act, which are to require that states supplied with nuclear materials from the United States should agree that all their nuclear installations are covered by IAEA safeguards. The trouble is that the act has demonstrated to all and sundry that arbitrary legislation can always interrupt supply. The result has been to damage confidence. The Anti-Proliferation Act should be repealed, but the IAEA meeting later this month should be used as a means of rebuilding confidence, if necessary by enabling the IAEA itself to become a supplier of nuclear fuel (as its existing statutes allow).

Dismal science in doghouse once more

Economists have often been derided for failing to agree among themselves on the future performance of this or that economy. Now they are in deeper trouble, and are being blamed for having misled the British government. Mrs Thatcher finds herself in serious political difficulties because it now seems that nobody has any clear idea of how much money there is at present in the British economy, or how quickly this money stock is increasing. The issue is important because the government has built its economic policy on an attempt to control both the rate at which the money stock increases and the rate at which the government itself borrows from the public. What has gone wrong?

Part of the difficulty is that the notion of what constitutes the money stock is, to say the least, abstract. It includes not merely the nominal value of the currency in circulation but also the value of borrowing from the commercial banks. This extension of the simplest concept of money makes sense for, in a modern state, bank money far outweighs currency in numerical terms. It is beyond dispute that the money supply is a valuable indicator of economic trends. Broadly speaking, the less money, the less there is to spend on consumption, investment or both. Moreover, governments (or their central banks) can hope to influence money supply by adjusting the interest rate at which they lend to commercial bankers. Monetarists and their critics differ only in their assessments of the importance of this instrument of monetary policy.

The Thatcher government, consistently (to its credit) but some say wrongly, has tried since its election in May last year to follow monetarist policies. In this spirit, it has kept interest rates high, and has tried to cut its own spending. It has been more successful at the first task than at the second — or so it seemed until the money stock was seen to have jumped by five per cent in July and a further three per cent in August.

The reasons for the sudden jump in the British money supply are linked simply with the abolition (in June) of a system whereby the Bank of England sought, two years ago, to limit the growth of money (currency and bank credit combined) by restricting the rate at which individual banks could increase lending. People seeking credit were forced at some cost to go to commercial companies which are not banks and also to banks abroad. Now that the

restrictions have been abolished, this informal lending has returned to the banking system, and the money supply has dramatically increased, causing widespread confusion and alarm. It remains to be seen whether the British government will be diverted from its present course by all the fuss, although the British Treasury last week was claiming that nothing had gone wrong, and that the "underlying" growth of the money supply was not excessive.

The Treasury may be right, but should know better than to peddle such facile nostrums. The money supply, although easily defined, is not easily measured. Bank money can be measured simply, but this is not the whole story. If, for example, a manufacturer persuades a supplier to give him an extra month to pay his bills, the money stock has been increased by that amount just as surely as if the manufacturer had borrowed from the bank. So the Bank of England's estimate is that attempts to control the money supply (as measured) will provoke a kind of monetary equivalent of Le Chatelier's principle — there will be compensating (but smaller) adjustments in the opposite direction.

Economists at the Treasury (and their academic colleagues) can be blamed for not having made this much crystal clear to the government, to politicians and to the public. Collectively they must also wear a hairshirt for having failed to emphasize that the control of the money supply is not an end in itself, but rather an index of how monetary policy as a whole is working. The British government's chief objective is to reduce the rate of inflation, to which end high interest rates (and reduced public expenditure) must surely contribute. A fall in money supply would indicate that inflationary pressures are abating. The converse is not however valid, as the past few months have shown. So there is every reason for the British government (if it has the nerve) to stick to its present policies, but with a more intelligent view of what the economic indicators mean. The most urgent need is, however, for a more intelligent (and effective) means of controlling public expenditure. Much of the British government's "saving" so far consists of the requirement that nationalized industries such as the telecommunications network should raise the capital they need for expansion from current users. The consequences are absurd — and inflationary to boot.

Polish scientists form free union

Polish scientists and university teachers have been quick to set up an independent trade union.

The Gdansk accords, signed on 31 August between Vice Premier Mieczyslaw Jagielski for the Polish Government and Mr Lech Walesa for the Joint Strike Committee of the Baltic littoral, guarantees to all Polish workers the right if they so choose to set up "free trade unions independent of Party and employees".

On 8 September, the new "Independent Union of Scientific, Technical and Educational Workers" (*Zwiazek Pracownikow Nauki, Techniki i Oswiaty* — ZPNTO) was established, and two days later it held its first delegate meeting, with 286 delegates (one for every 50 members) representing scientific and academic institutions throughout Poland.

During the labour unrest of the past two months, Polish scientists had kept a fairly low profile, although in at least two institutes of the Academy of Sciences — Experimental Biology, and Hydrology and Meteorology — meetings of support had been held to urge an agreement with the Joint Strike Committee.

The establishment of the ZPNTO is far more than an act of solidarity; the new chairman of ZPNTO, Zdzislaw Bibrowski, a researcher into energy problems at the Institute of Fundamental Problems of Technical Science of the Polish Academy of Sciences, considers that the Gdansk accords represent a new chance for Polish academic life to regain its autonomy, and freedom of research. "This is a problem of vital concern to all scientists," he said. Although ZPNTO hopes to act as a conventional trade union, defending the day-to-day interests of its members, its leaders see the issue of academic autonomy as all-important. If this is granted, they imply, many other problems will solve themselves. Already there are demands from the universities that rectors should be appointed by secret ballot and that senior appointments should be made on the basis of academic criteria only.

Such demands do not come only from ZPNTO. Last week the Party-linked Socialist Union of Polish Students published its own set of postulates, demanding greater autonomy for higher educational institutes, restoration of open discussion and a "moral revival" of the academic community. The governing board of the existing Union of Polish Teachers has adopted a resolution pledging itself to full democratization, independence and self-government; it seems in effect to have disestablished itself from the Central Council of Trade Unions. Addressing the start-of-session conference of university rectors last week, Janusz Gorski, Minister of Higher Education and Science, said that the postulates on higher

education put forward by the various academic establishments were now being studied, while Politburo member Andrzej Werblan urged public discussion on the issue of self-government in higher education as a basis for reforms. Even the Scientific Secretary of the Academy of Sciences, Dr Jan Kaczmarek, told a meeting of directors of research institutes that the academy will have to make a number of internal changes and redefine its role in the scientific community.

For those who have chosen the option of an independent trade union, however, the main practical problem at the moment is to get the new union registered as soon as the new legislation is through. Inevitably, there is considerable organizational work to be done, and ZPNTO is not attempting to rush things. The committee was elected by secret ballot at the delegate meeting and will hold office for only three months during the period of consolidation.

This committee, incidentally, represents a wide range of disciplines: two other physicists, Andrzej Ziabicki and Zbigniew Peradzynski, also from the Institute of Fundamental Problems of Technical Science, Tadeusz Klopotoski from the Institute of Biophysics and Biochemistry,

Krystyna Starczewska from the Institute of Philosophy and Sociology and Piotr Sasinski from the Institute of Literary Research. The only member of the committee not employed in one of the Academy's institutes is Viktor Kulerski, a school-master. The interim committee is largely Warsaw based, but Dr Bibrowski stressed that ZPNTO membership represents the academic community of the whole of Poland, and one of the main items discussed at the delegate meeting was the need to facilitate enrolment of academic workers outside the capital.

However, not all the Polish academic community is entirely happy about the foundation of ZPNTO. Last week's delegate meeting was held in the building of NOT (*Naczelna Organizacja Techniczna* — Chief Technical Organization) in Warsaw. When, however, the delegates and other supporters arrived (some 500 people in all), the NOT officials were unwilling to admit them. Only after lengthy negotiations were they allowed to go in, and even then only on condition that the chairman of NOT read a formal statement that NOT was acting merely as proprietor of the hall and accepted no responsibility for what might transpire. **Vera Rich**

DNA recombination forces resignation

Washington

Dr Samuel I.T. Kennedy last weekend resigned his post at the department of biology at the University of California, San Diego, in the wake of a critical report from the institution's biosafety committee. Dr Kennedy has been in hot water for the past several weeks, since it first became known that he had carried out cloning experiments with the Semliki Forest virus (SFV) at a time when cloning of the virus was prohibited under the recombinant DNA guidelines promulgated by the National Institutes of Health (NIH) (see *Nature*, 14 August).

Dr Kennedy has from the outset said that his work with Semliki Forest virus was accidental, and that he had been intending to clone fragments of the genome of another arbovirus, Sindbis virus. At one stage it had been suggested that the two kinds of viruses were confused when a package of vials containing various virus specimens was damaged during air transport from the United Kingdom.

The report prepared for NIH by the university's institutional biosafety committee now says that it does not accept Dr Kennedy's description of the experiments that were carried out, and suggests that the violation of the guidelines may have been deliberate.

This possibility, which has been strongly denied by Dr Kennedy, has led the committee to continue its ban on further

cloning experiments in his laboratory. It argues that agreements between research workers and biosafety committees must be based on mutual trust; and that in the absence of such trust "permission for cloning should not be granted".

The report contains two separate sections, one presenting a chronology of events as determined by the committee, the other the chronology as described by Dr Kennedy. Both descriptions agree on many points, in particular that subsequent laboratory tests revealed that cloning of SFV DNA had indeed occurred when it was prohibited by NIH guidelines (a prohibition that has since been lifted).

But there are significant differences in some of the details of the experiments and their timing. According to Dr Kennedy, defective interfering (DI) virus particles were prepared from stocks of Sindbis virus early in 1979 and subsequently used to generate further Sindbis DI particles. RNA prepared from the particles was cloned in a strain of *E. coli* in December 1979, and the DNA isolated from this clone was successfully used in January 1980 to transform mouse L cells.

The committee gives a slightly different version of events. It claims that it was a Semliki Forest virus preparation enriched in DI particles that was used in the summer of 1979 and that the DNA was used to infect cells in August and subsequently in January of this year. According to the

committee, it was only after the necessary methodology had been established and a number of different cloning strategies had been established that the successful cloning of the double-stranded DNA was carried out in March and early April — not last December, as Dr Kennedy claims.

The committee bases its reconstruction of the experiments on a close examination of Dr Kennedy's research notes, which it admits give a picture of him as an experimentalist with wide-ranging technical competence who describes protocols "clearly and completely".

However, it differs from Dr Kennedy in its interpretation of key sections of the notes. For example, it uses passing references to the use of SFV DI RNA material — which Dr Kennedy insists was used only for experiments establishing the conditions for complementary DNA synthesis — to support its conclusion that the experiments carried out in the summer of 1979 were specifically done to prepare SFV DI RNA for cloning.

The committee also bases its conclusion on the results of hybridization experiments carried out at the end of April 1980. Since both versions of the chronology agree that the clone should contain SFV sequences, the committee says that the RNA used to give a positive hybridization result must have come from SFV, quoting a published reference which claims a very low sequence homology between the two arboviruses.

Finally, with regard to the date of the cloning, the committee's conclusion that the experiments were carried out in March and April, and not last December, is based on its scepticism that a loose-leaf notebook purporting to describe the December experiments is in fact an accurate record, containing for example data transposed from the April experiments and other notebooks.

Dr Kennedy's resignation will relieve NIH of the need to decide what further action to take. Neither of the two previous violations of the guidelines has faced NIH with the problem of deciding which of two conflicting interpretations of events to believe.

Further light on the affair may eventually be shed by a confidential investigation being carried out by the university's biology department. This is expected to look into claims from laboratory workers that they knew last summer that they were working with material from Semliki Forest virus.

The departmental inquiry is also expected to investigate the source of the concern of graduate students that Dr Kennedy's work may have been in violation of the guidelines. This concern was reported to the head of the department in May, after a seminar given by Dr Kennedy at the Salk Institute was interpreted as confirming their earlier suspicions. (All four graduate students resigned from the department at the same time and have since been moved to other

departments in the university.)

The question has inevitably been raised of whether Dr Kennedy's action may have been prompted by the knowledge that a group at the European Molecular Biology Laboratory at Heidelberg is hard at work on the sequence of Semliki Forest virus. The German group was given the go-ahead to clone the virus under P3 conditions more than a year ago and in the past six months has been able to carry out subcloning of purified sequences under P2 conditions. As a result, the group has been able to sequence a substantial proportion of the virus genome.

Last Friday, Dr Kennedy resigned from the university, still protesting his innocence but complaining of "irreconcilable differences" between himself and "certain sectors of the university". Dr Kennedy said that he would shortly be sending his own version of events to NIH; and that he had resigned both as a matter of principle and because from a practical standpoint the decision of the institutional biosafety committee meant he would be unable to continue with his recombinant DNA research at the university, and "I don't want to settle for that".

David Dickson

Uranium enrichment

New French process

The French process for the enrichment of uranium by chemical techniques, under development since 1968, seems about to be launched commercially. The Commissariat à l'Energie Atomique (CEA) says that the process is now economically competitive with the diffusion process and that, because even small-scale plants can be economic, it may be attractive to countries planning only a small number of pressurized water reactors but anxious to secure a supply of enriched uranium for them.

The French, nevertheless, hold that for a number of technical reasons the new process will not increase the risks of nuclear proliferation. The CEA says that the Department of Energy in the United States has been studying the French process since September 1979, and that a decision on a cooperative commercial venture is expected soon.

The chemical enrichment process, inferred from the relevant patents, apparently involves the counter-current flow of aqueous and organic solutions containing uranium. The aqueous phase is a solution containing trivalent uranium ions. The organic phase contains uranium liganded to molecules rich in phosphorus-oxygen bonds. At equilibrium, it is claimed, the ratio of uranium-235 to uranium-239 isotopes may be up to a factor of 1.002.

Although the degree of separation obtainable from such a counter-current system is less than that from a single stage

of the diffusion process (estimated at 1.004), so that more stages would be needed for a given enrichment, the power requirements are much less. In the chemical process, energy is consumed chiefly in the interconversion of the aqueous and organic solutes. The CEA says that the overall power requirement is only a quarter of that involved in the diffusion process.

The CEA gives two simple reasons for believing that the chemical process would not contribute towards weapons proliferation. First, the time needed to bring a cascade of counter-current stages to equilibrium, which increases with the length of the enrichment cascade and thus the degree of enrichment required, might amount to ten years for a plant producing weapons-grade uranium. The CEA also says that the chemical process requires high uranium concentrations, and that problems of criticality would arise at high enrichment. In other words, people seeking to make weapons-grade uranium would risk blowing themselves up.

Not everybody is persuaded that the French process is as promising as the CEA says. On strictly economical grounds, gas centrifuges have much to offer. Like the chemical process, centrifuges (using gaseous uranium hexafluoride) work with near thermodynamic equilibrium, so that energy consumption is inherently low. Moreover, the degree of isotopic enrichment at each centrifuge stage in a cascade is much greater than that obtainable from diffusion plants and the French chemical process.

Inevitably, centrifuge plants designed to produce low-enrichment uranium can be quickly rearranged to produce a smaller amount of highly enriched uranium. Sceptics of French claims argue that the technical and economical advantages of the centrifuge design are so great that they will not be discarded for the nebulous political benefits of the French chemical process.

French interests are unlikely to be deterred by this argument, and are likely in future years to be impressing on Third World countries the benefits of a process that, in principle at least, offers independence from the uranium supply policies of the major powers.

Robert Walgate

Fast reactor

UK looks abroad

The shape of the possible collaboration on fast reactor development between the UK Atomic Energy Authority (UKAEA) and its opposite numbers elsewhere became clearer earlier this week with the publication of the authority's annual report for 1979-80 (HMSO, £2.00). Reports that the AEA might join in the exploitation of the French Super-Phénix design are confirmed, but the possibility of

collaboration with the United States remains. Negotiations are being carried on by governments, but the authority is hoping for a decision by the end of the year.

For the time being at least, the UKAEA takes the view that several countries (the United Kingdom included) have carried through the development of liquid-sodium fast reactors to the point at which commercial exploitation is possible. The objective of joining the Super-Phénix construction project (in which West Germany and Italy are already collaborating) would be to obtain the benefits that would flow from a joint plan for the exploitation of fast reactor technology in Europe, no doubt with agreements on "who builds what" as in the European collaborative aircraft projects.

Collaboration on Super-Phénix seems to have been made attractive by (among other things) a realistic appraisal of when it would be possible to build a commercial fast reactor in the United Kingdom. With the commitment (by the British government) to a public inquiry on the building of a first pressurized water reactor (PWR) by the Central Electricity Generating Board, and the shortage of qualified staff to carry out the necessary safety studies, the promised public inquiry on a commercial fast reactor could not begin until early 1985. Although a favourable outcome would allow construction to start towards the end of that year, the authority's fast reactor engineers will plainly have to kick their heels for quite a while if plans for collaboration come to nothing.

What does happen is for the British government to decide. Sir John Hill, chairman of the UKAEA, said this week that he looked for a commitment to the principle that fast reactors would be a primary source of energy in the next century, for a commitment (by the government) to a programme of fast reactor construction in the United Kingdom and for a decision about whether fast reactor development should be carried out in collaboration or otherwise. These questions, on the British government's plate since last December, may be settled by the end of the year.

One stumbling block will clearly be the cost to the British government of joining the European collaboration. According to Sir John, the French asking price is cal-

culated as 10 per cent of the estimated extra cost of Super-Phénix compared with a PWR of the same generating capacity, and works out as roughly £50 million. Sir John was, however, at pains to emphasize that negotiations with the French are still under way. No doubt the prospect of collaboration with the United States is being used as a counter in these negotiations, given the uncertainty about the American fast reactor programme.

For the rest, the UKAEA appears to be in robust, almost rude, health. The pilot-scale version of the advanced gas cooled reactor, which has been operating at Windscale since 1962, will cease to generate electricity next March and will afterwards be used for a series of tests of the safety of the design before being decommissioned. The AEA is plainly pleased with its new rapport with the British government — Sir John Hill this week contrasted its performance with that of other governments (mentioning Austria in particular).

The authority is also more aggressive about its safety record. Sir John says that he has written to the BBC to protest that the "public was badly misled" by the *Panorama* television programme on Windscale (*Nature*, 11 September), but acknowledges that, at Windscale, the authority might with advantage have started replacing some of its outmoded plant ten years ago (*Nature*, 7 August). Earlier this week, he was also regretting that "opposition by determined groups of people . . . exploiting ignorance and fear" has retarded the development and exploitation of nuclear power in many of the countries in which it is needed.

Pakistan uranium

Going ahead alone

Karachi

Pakistan seems to have taken a big step towards meeting its most urgent needs in the field of nuclear power production. After a closure of more than a year, the Karachi Nuclear Power Plant (KANUPP) has once again started producing nuclear electricity. KANUPP had to close down in mid-1979 after Canada — the supplier — unilaterally stopped supply of fuel and essential parts as a result of a dispute over Pakistan's bid to acquire a nuclear reprocessing plant from France.

The re-starting of KANUPP, a heavy-water reactor with a gross output of 137 MW, has been made possible by the fabrication of fuel bundles and some essential spare parts in Pakistan. The uranium has been mined locally, in the northern Dera Ghazi Khan District. There are believed to be ample reserves of uranium in Dera Ghazi Khan; preliminary investigations in the late 1960s revealed a reserve of at least 100,000 tons. The chairman of the Pakistan Atomic Energy Commission (PAEC), Mr Munir Ahmad Khan, is categorical in emphasizing that the

fuel bundles for KANUPP had been fabricated from locally mined ore.

Indigenous fuel fabrication has been on the cards since 1973, when Canada undertook to build a fabrication plant. Since the dispute with Canada in 1976, PAEC has designed and built a fuel fabrication plant at Chashma where the fuel bundles for KANUPP were fabricated.

Although the development of nuclear power has been in the melting pot for the past couple of years, the country now appears to be out of the woods. KANUPP, the only nuclear power plant in the country, has re-started. Another nuclear power plant of 600 MW output, to be installed at Chashma in the early 1980s, has once again been approved.

The chairman of PAEC says that consultants for the Chashma reactor project are to be appointed soon, after which bids for building the reactor will be invited. The cost of the reactor, he says, will be about \$800 million, compared with the original estimate of \$600 million.

The Chashma reactor, unlike KANUPP, is likely to use slightly enriched uranium fuel. The chairman of the commission, at a recent press conference, declined to comment on the development of enrichment facilities in Pakistan, saying that any utterance would be misinterpreted.

The President of Pakistan, General Zia-ul-Haq, is not, however, so diffident. He has declared that Pakistan has embarked on research on enrichment of uranium for peaceful purposes and to attain self-reliance.

Questions of the so-called "Islamic bomb" apart, there are good reasons to believe that Pakistan has been influenced on enrichment policy by the KANUPP troubles, and that it is now determined to be self-sufficient in whatever nuclear fuel its reactors need.

The search for self-sufficiency in enrichment may also be influenced by what is happening in the Middle East. The planners in Pakistan may have their eyes on the lucrative market for enriched fuel that may exist if Middle Eastern states install enriched uranium reactors of the types now being offered for sale. The planners seem chiefly interested in the centrifuge technology, which is less capital-intensive than the diffusion technology and which consumes less power. **Azim Kidwai**

US science policy

Press under pressure

Washington

The Office of Science and Technology Policy (OSTP, prop Dr Frank Press) has been sharply criticized in a report from the General Accounting Office (GAO). The essence of the criticism, resisted by Dr Press, is well expressed by the subtitle of the report, "Adaptation to a President's operating styles may conflict with con-



Uranium enrichment by centrifuges

gressionally-mandated assignments”.

The GAO is the investigative arm of Congress, and the report, published in Washington last week, was commissioned by Senator Adlai Stevenson, chairman of the Senate Commerce Committee's science, technology and space subcommittee. The GAO was asked to comment on how well OSTP was carrying out the legislative mandate that had been placed on it. OSTP was re-established by Congress in 1976 after having been disbanded by President Nixon four years earlier.

The general conclusion of the GAO report is that OSTP has operated effectively in many areas. But by seeking primarily to influence decision-making within the existing policy frameworks laid down by the White House, it has failed to provide sufficient independent leadership, as envisaged in the legislation, in laying out a broad national strategy for science and technology.

Dr Frank Press, director of OSTP and the President's Science Advisor, denies that his office has failed to fulfil its mandate. In a letter to the GAO he disputes the various charges made in the GAO report and claims that dealing with strategic planning issues through particular topics and areas has been the most sensible approach.

Two particular aspects of OSTP's activities were singled out by Mr Stevenson for the GAO's attention. The first was the question of how far OSTP had studied various issues related to the federal organization and management of science and technology policy listed explicitly in the 1976 Act.

Here the report says that many of OSTP's actions have addressed the issues defined, some extensively, such as interactions with the Office of Management and Budget over budget decisions, and relationships with other federal agencies such as the National Science Foundation.

It also points out specific requirements which have not been met, such as the provision of a regular report to Congress on the state of science and technology. The GAO quotes the opinion of OSTP staff that broad, comprehensive reports are less useful than reports and actions addressing specific current problems.

However, it is in response to Mr Stevenson's second question that the GAO makes its most critical comments and OSTP has reacted most sharply. This is the extent to which OSTP has been involved in strategic planning for US science and technology, and the constraints under which such planning has been conducted.

As GAO admits, the concept of strategic planning is difficult to define in the context of the decentralized and pluralistic market economy of the United States. But two ways in which science and technology are important to planning efforts are identified, first in their intrinsic role in

almost all policy issues and second in their increasing importance to society in their own right.

The GAO says that although OSTP staff attempt to give strategic perspective to considerations of topical or “mission” issues, such as energy or space, they believe that it is not feasible to do more comprehensive strategic planning while remaining effective within the Office of the President. OSTP therefore seldom studies the relationships of issues in the whole context of science and technology, the report says.

It characterizes this as largely a question of operating style. OSTP staff, says the GAO, “see themselves as an active agent of change within the system”, and feel that the office as a whole must respect the norms of the system, an attitude which the GAO says directly affects their ability to generate new issues or advocate change.

OSTP officials defend this strategy. They point out, for example, that apart from the advice offered on specific issues such as the reorganization of energy research or budget support for basic science, OSTP does not have any independent control over the policy-making process. And efforts at painting broad-brush policies might produce little result.

In his letter to the GAO, Dr Press comments that OSTP has taken what he calls a “sensible” course in its approach to strategic issues and that “we do not believe that our effort to focus policy development has been constraining. Rather it has enabled us to address policy issues on a manageable basis”.

He also reacts to the GAO's complaint that OSTP has no formal mechanism for establishing a broad agenda for itself by replying that this could be limited by the need for confidentiality at critical junctures in the policy process, and that “no one group is likely to have the breadth of view to encompass the ever-increasing span of science and technology”.

The charges made in the GAO report have been made in more piecemeal fashion from other directions in the past. OSTP has tended to dismiss many of these criticisms as reflecting a misinterpretation of the complexity of its role, and a lack of recognition of the constraints under which it has to operate.

In the short term the GAO report is not likely to have much effect. With less than four months of the current administration left, there is little pressure from the White House or OSTP for a change in policy; and Dr Press has made it clear that he is unlikely to stay on for another term.

More significant will be the role of the report in discussions over Dr Press's successor. Several members of Congress who have previously expressed similar conclusions — and are likely to do so again at a Senate hearing on the report, scheduled to take place on Friday — feel that this may be the most effective way to make their opinions felt.

David Dickson

Smallpox inquiry

Union in dispute

The repercussions of the smallpox accident at the Department of Medical Microbiology of the University of Birmingham, which led in 1978 to the death of Mrs Janet Parker, a technician in the department, were revived last week by the Association of Technical and Managerial Staffs (ASTMS). The union, which had been hoping to give evidence at the postponed inquest on Mrs Parker's death, due to reopen on Monday this week, has criticized the quality of the scientific evidence given at the earlier court case in which the University of Birmingham successfully defended itself against a prosecution brought by the Health and Safety Executive. The jury at the inquest gave a verdict of death by misadventure, rejecting the alternative of criminal negligence.

The ASTMS argument centres largely around the evidence given at the court proceedings by Professor Kevin McCarthy of the University of Liverpool, called by the University of Birmingham as an expert witness. His evidence was relevant because the official inquiry into the affair, conducted by a committee under Professor R.A. Shooter, concluded that Mrs Parker was most likely to have been infected with smallpox as a result of airborne transmission of the virus or, alternatively, by personal contact.

The import of McCarthy's evidence was that the procedures used in the smallpox laboratory at Birmingham were unlikely to have led to the production of aerosol contamination. To this end, he told the magistrates' court, he had himself carried out a series of experiments to assess the risk of aerosol production by procedures used in the smallpox laboratory. One of these involved the use of a water pump for actuating a pipette used for withdrawing supernatant fluid from Petri dishes in which smallpox virus had been cultivated. The pump was separated from the pipette by two flasks containing formalin.

Professor McCarthy's simulation of this equipment used the bacterium *Escherichia coli* rather than virus particles. On the basis of his results, he calculated that the chance of smallpox virus escaping in aerosol form would have been so small that one particle would get out once in 2,000 years.

ASTMS takes Professor McCarthy to task on the grounds that the simulation was not realistic. In practice, the ASTMS report says, the pipette would have been withdrawn from Petri dishes at frequent intervals, during which aerosol particles would be carried into the stream of water in the water pump and released into the laboratory by splashing.

The ASTMS document says that Professor McCarthy's evidence “tells us little except that he is a reasonably competent technician but does not really

understand the hazard". The document also criticizes as unwarranted Professor McCarthy's inferences about the production of aerosol particles carrying virus from safety cabinets used in the smallpox laboratory.

Those questioned earlier this week about the ASTMS allegations were unwilling to comment without an opportunity to study the text. Those witnesses at the court hearing who were reached by telephone said, however, that they stood by their evidence.

The coroner at the inquest on Mrs Parker's death had let it be known in advance that he did not wish to go over ground already covered at the court hearing. Mr Clive Jenkins, General Secretary of ASTMS, has nevertheless written to him with a copy of the document. He has also written to Dr Gerald Vaughan, Minister of Health, demanding that the Dangerous Pathogens Advisory Group be reconstituted by including public interest and trades union representatives, and that the government should make clear that it is prepared to spend the monies necessary to make laboratories in universities and hospital pathology departments safe.

Law of the Sea Hurdles to jump

Washington

Final agreement on international legislation covering deep-sea mining could prove more elusive than suggested by the successful completion of negotiations on a draft United Nations Law of the Sea Treaty in Geneva last month. Even if — as indeed seems likely — a draft treaty is finally agreed and signed by member nations after a final meeting in Caracas early next year, there remains the question of ratification, required of at least sixty states. And this could prove a major stumbling block, particularly in the US Senate.

Member companies and trade associations of the American Mining Congress (AMC) are meeting in San Francisco next weekend to decide their position on the draft treaty which emerged from the Geneva negotiations, the culmination of more than twenty years of talks.

Prior to the Geneva meeting, the congress had expressed strong reservations about some aspects of the US negotiating position, claiming that the chief US negotiator, Ambassador Elliott Richardson, was giving away too much in his eagerness to get the treaty signed.

Many of the companies still express the same reservations. And the AMC's views could be crucial in determining whether the US Congress decides to back the treaty. Earlier this year, following strong pressure from the mining companies, Congress passed a unilateral law defining conditions under which US companies could engage experimentally in deep-sea mining from

1981 unless the UN treaty is in force. Similar actions by Germany, and proposed actions by the United Kingdom and France, are among the factors said to have precipitated the Geneva agreement.

The stakes are high. Total investments of more than \$1,000 million are expected to be made by each of the four major consortia now preparing to engage in deep-sea mining, primarily for manganese nodules containing nickel, cobalt and copper. The companies are demanding, above all, a degree of certainty about a reasonable return on their investment.

Among the areas that the mining companies are unhappy about are:

- The lack of guaranteed representation of the United States on the 37-member council of the International Seabed Authority, even though the Soviet Union is given three seats, a position which one executive predicted that the Senate would find difficult to accept.

- The participation as signatories of the treaty of organizations other than UN member states, such as the Palestine Liberation Organization. This could entitle such organizations to share in the profits of deep-sea mining which the US companies are required to provide to the authority for distribution as development aid.

- The requirement that mining companies should make their mining technology available to other countries who demand it.

The major question now facing both US and UN negotiators is whether any movement is possible on these issues before the treaty is signed next year and, if not, whether they are likely to provide a sufficient obstacle to prevent Senate ratification, given the many other advantages to the United States in an international treaty that covers all aspects of nations' rights to the oceans.

None of these reservations is, in itself, expected to be a major stumbling block to the final treaty. Nor, among those who are strong supporters of the treaty, is there any desire to open up discussion again in case the whole package begins to unravel. But claims that "it is all over bar the shouting" may turn out to be a little premature.

David Dickson

Overseas students

Some win, some lose

British universities and colleges appear to differ markedly in their expectations of numbers of overseas students in the coming academic year. Some universities — a little to their surprise — expect there will be very little change between last year and this in the numbers of students from overseas. Others expect that numbers will be, by contrast, substantially reduced.

The issue has become a lively topic of conversation among academic registrars because the coming academic year is the first in which the steep increases of student fees decreed by the British government a

year ago will begin to bite. The fees apply only to new students, or to students embarking on new courses. Registrars are also quick to point out that at this stage in the academic year, before students have actually registered and paid their fees, estimates of how many will turn up are bound to be provisional.

The universities where there appears to have been very little change in the demand for places include the University of Salford and Loughborough University of Technology. At both institutions (as at most universities) overseas students are now required to produce not merely their fees but a financial guarantee from a bank or some scholarship awarding body, which may require that a student should have £6,500 at his disposal in each of three consecutive academic years.

Elsewhere, the preliminary figures suggest that some kinds of courses are likely to suffer more than others. Applications for places on MSc courses appear to be comparable with last year's at some institutions, which are nevertheless guessing that overseas students will be reduced by ten per cent or more in undergraduate and PhD courses.

One of the difficulties of estimation is that students who have nominally accepted places fail to turn up by the beginning of term, for most British universities early in October. In normal years, it is expected that overseas students will apply to several universities but in the end accept a place at only one of them, which means that a certain amount of double counting is unavoidable at this stage in the academic year. But some also fail to turn up for reasons which are never explained. Some registrars fear that difficulties over finance may increase the numbers in this category in the coming year.

With all these reservations, Imperial College, London says that there has been a "discernible" reduction of the numbers of completed applications from overseas. Portsmouth Polytechnic, one of the largest institutions of higher education in the United Kingdom, is similarly affected — and wonders ruefully whether the explanation may not be the rules which require polytechnics to charge fees laid down by the Department of Education and Science. This year, polytechnic fees for overseas students are nominally some £300 to £400 greater than those in universities.

While registrars at all kinds of institutions agree that the present period is more confusing than any other within living memory, most of them appear to be mildly encouraged that the decline in numbers has not been as dramatic as some of them were fearing earlier this year. Unless there is a sharp deterioration in the next few weeks, overseas student numbers are unlikely to be reduced by anything like the 50 per cent feared by some or by the 30 per cent which appears to have been the base-line for financial planning for the coming year.

CORRESPONDENCE

Pay as you go

SIR—Although far more interesting and important things are now happening in Poland, let me bring to your attention one more Polish subject connected with the Polish regime's abuses of decency or simply the law. Every Polish scientist going abroad for professional purposes, and for a long period, is forced to sign a declaration. ("Long period" means more than eleven weeks. Why just eleven? — Nobody knows.)

The declaration is an obligation to pay some arbitrarily fixed sum of money every month to the so-called "hard currency scholarship fund", created apparently to finance foreign trips for some unspecified persons. The point is that although many Polish scientists were forced to, and did, pay their money to the fund, no-one has ever heard of anyone actually getting something from this fund. There are rumours that the money is being used to finance "study" trips for Polish journalists, for example to the United States, where they use the American money to collect materials for painting the American system in black colours. Indeed, the fund in question is under no form of public control, and no-one really knows to what purpose the money is used. The character of the deal comes out most clearly from this paragraph in the declaration:

"If the currency regulations of the country where I shall be staying would make it impossible to send my contribution to the hard-currency scholarship fund via a bank, I undertake to send the remittance to the nearest agency of the Polish People's Republic abroad."

As you are aware of the political situation in Poland I hope that you will understand my request to keep my name, and address a secret.

Name and address withheld — Ed, *Nature*

Nuclear power costs

SIR—The Central Electricity Generating Board (CEGB) is to be congratulated on providing more details of its cost comparisons for the different power station types. As it emphasizes in its report, current relative economic performance of coal and nuclear systems is not an adequate guide to future investment decisions. However, one could argue that operating experience is a guide to future technical (rather than economic) performance. Load factors are one measure of technical performance, and your report made some pertinent observations regarding the reported load factors of power stations operated and planned by the CEGB (*Nature* 21 August, p.753). Obviously the cost per kWh per annum is sensitive to the load factor and it is informative to recalculate the predicted generating costs of possible future nuclear and coal power stations using load factors consistent with those quoted for existing stations. The table summarizes factors on design output given in the CEGB report load.

	Nuclear	Coal
Stations (1965–77)	56%	61%
Hinkley B; Drax I	43%	73%
Stations under construction	49%	56%
Future stations	63%	54%

The figures show an unexplained optimism for nuclear systems and a corresponding pessimism for coal systems. Assuming load factors consistent with experience (say 70% for coal and 50% for nuclear) reverses and predicted cost ranking for the fuels in question. Specifically, nuclear comes out some 8% more expensive than coal (2.8 p per kWh compared with 2.6 p per kWh) rather than 35% cheaper as derived by the CEGB.

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RICHARD MARSHALL

Why examine?

SIR—It was interesting to read the article on school examinations (*Nature* 3 July p.2). As one who, although born and educated in the United Kingdom, has spent most of my working life teaching overseas, my usual complaint is that the schools we have to deal with do not teach A levels and that it is so much better in the UK where they do. The main reason why the schools I know do not teach A levels is not choice, but simply lack of qualified teachers.

As in everything else, there are pros and cons and things look different from the other side of the fence. One thing I am convinced of, however, is that good schools are much better at teaching A-level material than are universities. In general, university staff do not make good school teachers.

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Third World matters

SIR—"Pesticides in developing countries" (*Nature* 28 August p.832) was a patronizing editorial on the *New Internationalist*; a journal that is not a "citadel for extreme environmentalists" but a medium for exposing injustice, stimulating debate and campaigning for and on behalf of the poor of the Third World.

Transnational corporations are mostly the villains because it is often their trading practices that accentuate North-South differences. Global profit maximization policies tend not to be compatible with the needs of the people of the Third World. For example, infant mortality rates have increased significantly in areas where advertising has persuaded mothers to abandon breast feeding for Nestle milk powder products. Other cases are more insidious: uncontrolled marketing of harmful products, including cigarettes and pesticides. High blood levels of DDT (30× that in USA) being the corollary of one aggressive marketing campaign in Guatemala.

New Internationalist is not a scientific journal. It does try to report facts accurately and from an objective stance. This is expected of *Nature*. It was unfortunate that a basic, scientific definition had to be distorted in order to accommodate editorial views: DDT is a poison, a poison being a substance that when introduced into or absorbed by a living organism (or ecosystem) destroys life or injures health.

May I reiterate Clive Jenkins' final point (*Nature* 21 August p.754) that *Nature* should abandon prejudice and support quite reasonable crusades to protect people.

PETER CLARKE

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Felig replies

SIR,—Your account of the events surrounding my resignation from Columbia University (*Nature*, 21 August) contains a number of inaccuracies and omissions which may lead the reader to a distorted view of this unfortunate affair.

Firstly, my proposal that Dr Soman be considered for a faculty appointment at Columbia occurred in January rather than February 1980, before the first audit which revealed data falsification by Dr Soman. Secondly, the letter by Soman in February 1980, declaring his responsibility for the similarities to the paper from the Roth laboratory and for "fudging" data, was not written at my request but was his own effort to "set the record straight" at the time of his resignation. Dr Soman had, in fact, admitted in March 1979 that he had retained a copy of the Wachslight-Rodbard manuscript and had used parts of it to prepare his own manuscript. At that time he was severely reprimanded and steps were taken to assure Roth's priority.

Thirdly, the delay in obtaining the first audit was a result of the failure of the agreed upon auditor (Dr Edward Rall) to follow through with his offer to come to Yale and review the original data. Fourthly, while Dr Roth is quoted as considering it "careless" for us to allow publication of our paper pending an audit, he had in fact written to us on 5 March 1979 that "I hope you will go on to have your fine and original piece of research published appropriately". Fifthly, those papers which were considered questionable after the second audit involved studies for which original data were not available for audit because Dr Soman (by his written admission) had destroyed the original notebooks. Sixthly, in addition to informing Dean Tapley on 27 February 1980, I met with five of the senior faculty in the Department of Medicine at Columbia in May 1980, prior to my assuming the Chairmanship, and informed them of Soman's data falsification and that I would be sending letters of retraction to journals.

Finally, your article fails to take note of the fact that at the time the paper on anorexia nervosa was submitted for publication by Soman and myself, Soman was in his third year of a faculty appointment at Yale, had extensive independent grant support, was responsible for his own laboratory and was highly regarded by all his colleagues. Any retrospective analysis of the handling of his wrongdoing in using the Wachslight-Rodbard manuscript to prepare his own must be viewed in that context.

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NEWS AND VIEWS

What we don't know about pain

from P. D. Wall and C. J. Woolf

A RECENT article in these columns¹ by Chung and Dickenson discusses the relationship between pain, the enkephalins and acupuncture. We are told that "placing a single needle into a certain point of the body can produce . . . a potent amelioration of pain". It is proposed that acupuncture analgesia acts by the release of endogenous opioids within the brain and that these enkephalins indirectly stop "pain cells" in the spinal cord from transmitting their messages. How one wishes all this were true. If it were so, we would be well on our way to understanding and controlling the chronic pain states which continue to plague so many. We would like to supplement Chung and Dickenson by examining whether acupuncture does produce pain relief, what evidence there is for a role for the endogenous opioids in modifying nociceptive transmission and how the nervous system is organized to transmit and process information about noxious stimuli.

After 2,000 years of continuous use in China and some 350 years of intermittent Western enthusiasm, we still await controlled clinical trials which conclude that acupuncture is effective in treating chronic pain². Innumerable, uncontrolled and anecdotal reports exist³. Such 'trials' report success rates of up to 94%⁴ but in a controlled trial at the University of Washington Pain Clinic⁵, none of a 100 patients showed any objective benefit after three weeks of acupuncture therapy. Another controlled trial found that acupuncture does produce a very transient analgesia, but that this analgesia occurs equally with 'sham-acupuncture' and with needling of the traditional meridian points⁶. For acute pain, we read of the successful application in China of acupuncture in half a million operations⁷, but this does not match the experience of an invited team of western experts^{2,8}, who witnessed operations on highly selected patients, only a small fraction of whom had pain free operations with needles alone. Moreover the use of acupuncture for surgery in China is reported to be steadily declining^{2,9}. There is no doubt that powerful analgesia with acupuncture does occur and has to be explained. Unfortunately, it is a rare phenomenon and for the huge majority of patients the needle is a blunt tool. Evidently acupuncture, when it works, acts by some mechanism which does not always operate in the majority of people, unlike the uniform ability to react to narcotic analgesics.

All sensory stimuli interact with each other producing distraction, jamming and specific local and distant interactions. Many have selected special examples of this general phenomenon and called them 'experimental acupuncture' although they usually bore no relationship to traditional acupuncture practice. In healthy volunteers, needling or electrical stimulation can produce a mild rise in the threshold for certain painful stimuli^{10,11}. This antinociceptive effect is most marked in the region of the painful stimulus¹³ and not at a distance or at 'meridians' as predicted by classical acupuncture. The analgesia produced by such experimental acupuncture is quite feeble and unlikely to be clinically useful, and must not be confused with the rare cases of perfect surgical analgesia produced by acupuncture or with the use of segmental transcutaneous electrical stimuli which have a highly satisfactory effect on certain types of hyperalgesias¹²⁻¹⁴ with minimal effects on cutaneous pain thresholds¹⁵.

There is no doubt that the enkephalins and a number of other opioid peptides are highly concentrated in specific regions within the brain and spinal cord¹⁶, but their role, if any, in pain remains an enigma. One attempt to study this problem has been to investigate the effect of naloxone, an opiate receptor antagonist, on a variety of different pain states and analgesic procedures. Naloxone completely reverses the analgesic effects of administered narcotics¹⁷. If the endogenous opioids were active in normal men or animals one would expect that naloxone would antagonize their action and consequently produce pain or a drop in pain thresholds. Most studies in man have shown that naloxone has no effect^{18,19}, while in animals naloxone has been found to produce hyperalgesia^{20,21}, analgesia²², and no change²³, in the response to noxious stimuli. Patients suffering from chronic pain are unaffected by naloxone although one might expect these patients to be mobilizing whatever anti-pain resources they possess²⁴.

Naloxone has been reported to produce a partial reduction both of experimental acupuncture analgesia in man²⁵, and of the analgesia produced by midbrain electrical

stimulation²⁶, but these results need to be confirmed since conflicting reports are found in animal studies^{27,28}. There is evidence in the rat that local segmental interactions between sensory inputs in the spinal cord are modified by naloxone^{29,30} but it will be difficult to determine whether similar changes occur in man. Recently, in fascinating but highly controversial work, Levine & Fields³¹ report that very high doses of naloxone abolish the placebo reaction, suggesting that the 'analgesia' resulting from blank injections may be produced by the mobilization of endogenous opioids.

Whatever function the opioids do perform in pain, it is unlikely to be simple as indicated by the growing list of endogenous peptide compounds with opioid activity and the subdivision of the opiate receptors into numerous functional categories. One of the problems in interpreting studies using naloxone is that it has a greater affinity for some classes of opiate receptors than for others³². Nevertheless it is clear that there is no evidence for an internal narcotic dispensing system in the brain automatically controlling the intensity of perceived pain.

In their summary of the anatomy and physiology of the pathways which bring news of injury to the brain, Chung and Dickenson use the most classical model for simplicity and brevity. They refer to pain fibres and pain cells in peripheral nerves and spinal cord as though there is a specialised chain of nerve cells which leads inevitably from the fact of injury to the sensory experience of pain. They further isolate this 'pain system' by saying that "in stark contrast to the other senses it is characterised by elaborate feedback networks" which "ensure that it is fail-safe" and "which, when activated, reduces the intensity of persistent pain". This approach "which implies a distinct anatomical and physiological pain system separate from other sensory systems" represents a definite philosophy which would shape future experiments and have considerable therapeutic implications if true. For example, given a distinct anatomy such as the pain cells described and a distinct feedback control such as the raphe nucleus and distinct chemistry such as the enkephalins, one might reasonably hope to achieve pain control with no influence on other aspects of feeling or behaviour. The concept of a separate pain system originates in our own introspective certainty that pain itself is a clearly discrete category of experience which we are convinced implies

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injury. Therefore we are also convinced that we must possess an isolated private alarm system which inevitably links injury to pain. The failure of this concept to explain the observed clinical and experimental facts has been discussed extensively and led to the gate control theory³³ and its extensions³⁴. Chung and Dickenson use gate theory³³ as though it applied only to a 'pain system'. An acceptable pain theory would have to incorporate an explanation of at least the following clinical facts: (1) the bizarre coupling of the amount of injury to the amount of pain, (2) the triggering of pain by innocuous stimuli to normal tissue (tenderness, referred pain and trigeminal neuralgia), (3) the sensation of pain in missing limbs, (phantom pain), (4) the failure of narcotics to influence some pains, (brachial plexus avulsions), (5) the success and failure of the various counter-stimulation methods (acupuncture, transcutaneous stimulation).

Undoubtedly for lack of space Chung and Dickenson have concentrated on only one particular 'pain' inhibitory system, which is said to operate via noxious inputs and the nucleus raphe magnus to modify the activity of spinal cord nociceptive cells. However, as Chung and Dickenson point out this is only one example of many such inhibitory systems³⁵. The brain has many reasons, tactics and strategies for selecting and modifying the incoming information it receives. Each of the different neural systems responsible for such 'controls' offers explanations for particular pain states, and opportunities for therapy. Some of the control systems are located in the spinal cord. For example, stimulation of large low threshold sensory fibres produces a striking inhibition of the response of spinal cord cells to peripheral injury³⁶. Other control systems act via the brain³⁷ and there is good reason to suspect that some of these inhibitory effects are mediated by the small cells of the substantia gelatinosa³⁸. Therefore the activity of all nociceptive cells is controlled by a balance of excitatory and inhibitory mechanisms mediated by many neural circuits, some local within the spinal cord and some with long arcs extending from cord to brain.

Experimental investigations of the functional profiles of neurones responding to noxious inputs and of their 'controlling' influences have been performed either in isolated parts of the nervous system such as the spinal cord or in highly unphysiological preparations such as anaesthetized or decerebrate animals. These experimental preparations represent a study of the nervous system locked at one extreme of its functional repertoire. While defining limits is a responsible scientific pursuit, it is difficult to establish the validity of these data in speculating about the operation of the system in the behaving organism. Having studied the functional repertoire of cells in these artificial states, it is clearly

necessary to return to a study of their behaviour in the intact behaving organism before we can label cells as 'pain' cells. We are a long way from that step. How best to approach it? One way is to return to the animal and human patient to define the input-output nature of their problems rather than to force them to conform with ancient theory of how they ought to behave. We are emerging from a necessary era of physiology in which attention was concentrated on sudden stimuli and the consequent surge of impulses which ran through the nervous system over the subsequent 100 milliseconds. These volleys are travelling over neural circuits whose transmitting qualities have been set by much slower processes. These began in the embryo and obviously depend on a relatively fixed anatomy but it is now becoming apparent that there are also plastic physiological and biochemical processes which shape the responsiveness of nerve cells. It may well be that the huge newly discovered family of peptides including the enkephalins and endorphins have more to do with setting the mode of operation of the nervous system's analysis mechanisms than with the incoming messages which are to be analysed.

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Chung and Dickenson comment: There are two separate issues concerning acupuncture. First, we must ask if acupuncture analgesia is a reproducible phenomenon that can be studied in the laboratory. This question can be evaluated as we have done¹, from published studies. Second, we must ask if acupuncture is clinically effective. This is more difficult to assess, as there are

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numerous claims and counter-claims and the arguments are generally construed by carefully selecting one set of reports from the 'western experts' and dismissing the results which do not conform with preconceived opinions. We feel that controversy at present over the validity of clinical data is unproductive. Except for a brief mention that stimulation described by the experimenters as 'acupuncture-like' (as distinct from transcutaneous electrical stimulation) appears to be especially effective in relieving chronic pain in man²⁻⁵, we avoided this issue in our review. There are however, many studies demonstrating that electroacupuncture and similar manoeuvres in animals enhance the threshold to pain and influence the responsiveness of pain cells. For example, cells responding steadily to painful stimuli are silenced when "acupuncture-like electrical stimuli" are delivered elsewhere on the body⁶. How the reduced activity of these cells is related to the subjective intensity of pain is unknown, as are the precise neural mechanisms by which such an inhibitory influence is brought about. Regardless of its ultimate clinical usefulness, the phenomenon deserves further investigation, and much of the current research into pain is indeed aimed at elucidating the mechanisms mediating inhibitory influences.

As Wall and Woolf point out, the involvement of opioids in modulating pain is not fully understood and conflicting evidence exists. It is likely that the role of opiates or endogenous peptides in the perception of pain will turn out to be complex. Of the many levels of the central nervous system at which pain transmission can be modulated, the raphe-spinal system appears to exert the most powerful inhibitory effects. We concentrated on this system mainly because the evidence for its inhibitory role is least controversial. Opiates⁷, central electrical stimulation^{8,9}, acupuncture¹⁰ and, more recently, transcutaneous nerve stimulation¹¹ have been claimed to act to some extent via this nucleus in producing their analgesic effects.

We have not stated, nor implied, that the pain system is isolated from "feelings and behaviour" or from other sensory systems. There are chains of cells which respond preferentially, and often solely and exclusively, to what the experimenter would consider to be noxious stimuli. These are usually referred to as 'cells which are involved in transmitting nociceptive messages', but to avoid circumlocution, we called them 'pain cells'.

1. Chung & Dickenson *Nature* **283**, 243 (1980).
2. Sjolund & Eriksson *Brain Res.* **173**, 295 (1979).
3. Andersson *et al. Brain Res.* **63**, 393 (1973).
4. Boureau *et al. Elect. Clin. Neurol.* **47**, 322 (1979).
5. Melzack *Pain* **1**, 357 (1975).
6. Department of Physiology, Kirin Medical College, Changchun. *Scientia Sinica* **20**, 485 (1977).
7. Dickenson *et al. Brain Res.* **170**, 95 (1979).
8. Taub *Exptl. Neurol.* **10**, 357 (1964).
9. Oliveras *et al. Brain Res.* **120**, 221 (1977).
10. Du & Chao *Scientia Sinica* **19**, 137 (1976).
11. Woolf *et al. Pain* **8**, 237 (1980).

Hopping and running on two legs or four

from R. McNeill Alexander

MOST mammals run on all fours but some run on just two legs (like men) or hop (like kangaroos). Some birds run, some hop and some do both. What are the relative merits of the different gaits? Do they differ in energy cost?

Energy costs of running and hopping can be inferred from measurements of oxygen consumption. Some of the difficulties of physiological experimentation on moving animals can be avoided by training the animal to run on a moving belt as shown. Data has been collected for mammals ranging from mice to horses and lions, and for many birds (summarized by Fedak & Seeherman *Nature* 282, 713; 1979). Nearly all of it conforms to a simple rule: the power P required for level running at speed u is given by

$$P(u) = P(0) + ku \quad (1)$$

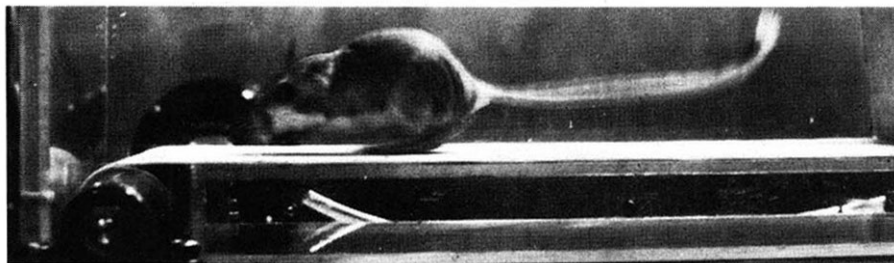
where k is a constant for the particular animal. $P(0)$ is a little more than the power consumption at rest because it includes a component for maintenance of posture.

Equation (1) implies that the energy required to travel a given distance (in excess of the requirement for standing still) is more or less independent of speed.

This may seem remarkable, but experiments with red kangaroos gave an even more remarkable result (Dawson & Taylor *Nature* 246, 313; 1973). The power consumption of a hopping kangaroo is independent of speed, or may even fall slightly as speed increases. The energy used by a kangaroo travelling a given distance therefore falls steeply as speed increases. At low speeds a kangaroo uses far more power than (for instance) a gazelle of the same mass, but at 5 m s⁻¹ the two animals use about equal powers. It has been suggested that at higher speeds, a kangaroo might travel more economically than a gazelle, but it is difficult to measure power consumption at high speeds because the animals build up oxygen debts.

Various rodents hop like small kangaroos. There are kangaroo rats (*Dipodomys*) in N. America, springhares (*Pedetes*) in Africa and hopping mice (*Notomys*) in Australia. Is power independent of speed for them all? Thompson, McMillen, Burke & Taylor (this issue of *Nature*, p223) find that it is not. Equation (1) holds for many and probably all hopping rodents, with values of k about the same as for quadrupeds of similar mass. The same is true for the rat kangaroo (*Bettongia*, a marsupial). The large kangaroos seem to stand alone in their peculiar energetics.

Though equation (1) holds for a wide variety of animals, the constant k is



Dipodomys deserti hopping on a moving belt to assess energy consumption. Experimental details are given by Thompson *et al.* on p.223 of this issue of *Nature*.

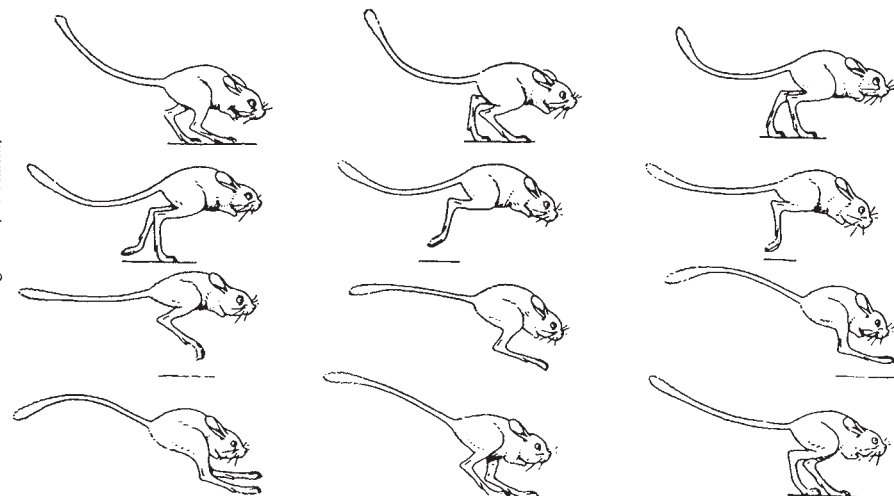
different for animals of different sizes. Until recently the data seemed to indicate that k was proportional to (body mass)^{0.6} for quadrupeds but to (body mass)^{0.8} for bipeds. Consequently bipedalism was more economical than quadrupedalism for small animals, but less economical for large ones. Why, then, are there small quadrupeds (such as mice) and large bipeds (such as ostriches and, formerly, tyrannosaurs)?

This apparent paradox seems to have been an artefact of the sample of species originally selected for study. Fedak & Seeherman (*Nature* 282, 713; 1979) showed that though penguins and geese need twice as much power for running as dogs of the same mass, ostriches are as economical as ponies. For any body mass, a wide range of values of k is possible, but the general trend both for quadrupeds and for bipeds is for k to be proportional to (body mass)^{0.7}.

It would be satisfying to be able to explain why kangaroos and smaller hoppers have different relationships between power and speed, and why k tends to be proportional to (body mass)^{0.7}. This has not yet been achieved. Calculations based on the mechanical work required of the leg muscles provide satisfactory explanations of the power requirements of kangaroos and also of quadrupedal mammals of similar size, and show that slow hopping is more expensive than

running because it involves larger fluctuations of potential energy (Alexander & Goldspink *Mechanics and energetics of animal locomotion*, 1977).

Similar calculations grossly underestimate the power requirements of small runners and hoppers, such as mice and quail: indeed they predict that k should be proportional to (body mass)^{1.0}. Taylor, Heglund, McMahon & Looney (*J. exp. Biol.* 86, 9; 1980) have recently tried to resolve this discrepancy, by suggesting that the main metabolic energy cost of running is associated with the generation of muscular force, rather than the performance of work. The forces required are proportional to body mass but smaller mammals need faster muscles, with shorter cross-bridge cycling times, so exertion of force may be more expensive for them. Taylor and his colleagues show for rats, dogs, men and horses, that carrying a load increases k in the same proportion as it increases the forces on the feet. This is consistent with their hypothesis but is not clear evidence for it (as they themselves admit), because the experiment increases forces and work in the same proportion. The hypothesis is unlikely to win general acceptance unless it can be supported by less ambiguous evidence and shown to be quantitatively consistent with our knowledge of muscle physiology. □



One of the hopping rodents (a jerboa, *Allactaga severtzovi*). The action is like a kangaroo but the energetics may be quite different.

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from *How mammals run* Kater Publishing House, Jerusalem, 1974.

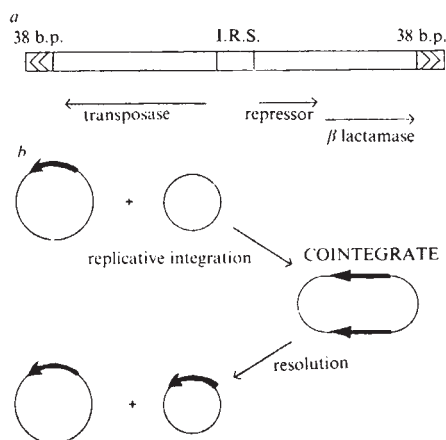
Movable genes

from Ron McKay

BARBARA McClintock's early studies on the breakage-fusion bridge cycle in maize served for many years as a reminder that a major class of genetic change was not explained by the central theories. This year's Cold Spring Harbor Symposium (May 28 — June 4, 1980) showed quite clearly that genetic instability now occupies a central place in our understanding of both heredity and development. The recent rekindling of interest in this field stemmed from observations of the rapid spread of drug resistance markers in bacteria but now extends to eukaryote molecular genetics. In prokaryotes it is clear that there is a class of DNA which promotes its own rapid spread within and between bacterial species. The physical structure of these mobile genetic elements is well understood and current goals are to determine the mechanism and enzymology of this genetic exchange.

One of the best understood of the mobile elements is the bacterial transposon Tn3. Transposon Tn3 confers resistance to the antibiotic ampicillin as it carries a gene which specifies the ampicillin degrading enzyme β -lactamase. The studies of Heffron, Casadaban and others have shown that as well as this genetic marker Tn3 carries two genes which function in trans to promote genetic movement or transposition (Fig. a). After transposition a new copy of Tn3 is found at a new integration site but the old copy is still found at the original site — a key feature which distinguishes the movement of transposons from the integration-excision system found in bacteriophage lambda. However, as with phage lambda, transposable elements require certain DNA sequences in cis for transposition. These sequences fall within the inverted terminal repeated sequence which is found at the ends of all mobile genetic elements. The process by which a new copy of a transposable element coding for its own transposase is inserted into another replicon is thought to occur via a fused structure called a co-integrate in which two copies of the transposable element flank the donor molecule (Fig. b). Cointegrates from transposons known to code for their own transposases (Tn3 and Tn5) have only been found using mutant transposons. Normally no cointegrates are seen but in some mutations all the transposons are cointegrates. Therefore cointegrates have been proposed as intermediates in transposition. Other transposons which have not been shown to code for transposases but which have IS1 elements at their ends (for example, Tn9) give rise to cointegrates as the end product of wild type transposition. The fused replicon can be resolved, as shown in Fig.

b, to give the two starting replicons with an additional copy of the transposable function. Heffron (University of California, San Francisco) has shown that the resolution of co-integrates also requires a DNA sequence within Tn3 in cis. This sequence falls in the central region of Tn3 and is called by Heffron the internal resolution site (IRS). The DNA sequence of IRS shows some similarity to the terminally repeated DNA sequences but the IRS sequence is unable to substitute for a terminal repeat in transposition. So here we have clear evidence that there is another sequence required in cis for transposition to occur. In addition to IRS there is a resolution function which may be supplied



(a) Structure of transposon Tn3, (b) the transposable element is inserted into another replicon via a cointegrate.

in trans. This function was shown by Sherratt (Glasgow University) to be coded by Tn3. Sherratt's studies shows the resolution function to map close to a gene whose product is known to repress transposition. Whether the resolution of co-integrates is another function of the previously defined repressor is not known. Resolution of co-integrates may be a site specific recombination catalysed at a high frequency by two copies of IRS and repressor.

Genetic studies of Tn3 have shown that the rate of transposition is influenced by genes carried by Tn3. Biek (Utah University) has used transposon Tn5 to investigate another feature of regulation. In transductional crosses a Tn5 element in the recipient cell inhibits transposition of the incoming Tn5. These results are consistent with a Tn5 coded repressor of transposition. Genetic mapping localised the inhibition close to the Tn5 drug resistance marker, neomycin phosphotransferase. However, the two functions are distinct since nonsense mutants of the *neo* gene do not affect transposition. The DNA sequence of Tn5 has been established (Auerswald, Heidelberg University) and the transcription and translation products identified (Reznikoff, University of Wisconsin), so we can soon expect to know the molecular basis of Tn5 mediated immunity.

The terminal inverted repeats of Tn5 are

much larger than those of Tn3 and Reznikoff's laboratory has shown that each arm codes for two polypeptides but the molecular weights of the two proteins coded by each arm differs. Genetic studies have also shown that the two arms are not homologous. Mutations in the repeat proximal to the *neo* gene do not affect transposition but mutation of the distal arm can prevent transposition. The reason for this is apparent now that the DNA sequence of Tn5 is known. The two arms differ by only a single base in 1,450 base pairs but this generates a stop codon in the coding sequence of the repeat proximal to the *neo* gene so bringing about the altered proteins and the lack of function. Grindley (Yale University) generated variants of the similarly organised transposon Tn903 which have deletions in one arm. He then mutated the other inverted repeat by inserting 10 base pair Bam HI linkers at suitable restriction sites. This method of *in vitro* mutagenesis, extensively used by Heffron, generates frame shift mutations as the insertion is not an integer multiple of the three base codon. Grindley found that most double mutants of Tn903 had lost the ability to transpose. The one exception which still transposed had suffered only a 9 base insertion, presumably leaving the distal part of a gene in phase. Although this is good genetic evidence for the expression of a gene in the inverted repeats of Tn903 no protein has yet been found. How this expression leads to transposition is still not clear.

In Tn5, Tn903, Tn3 and in bacteriophage Mu genetic and structural studies have identified genes and in some cases gene products which must mediate and regulate transposition. No *in vitro* assay for transposition is available, nor has enzymatic activity been associated with any of the putative transposases.

How does transposition occur? There may not be a single answer to this question. For example IS4, unlike most transposable elements, is site specific. Quite rightly these exceptions do not prevent the search for a single unifying model. As it is generally accepted that a copy of a transposon remains at the original site when transposition occurs, and because replication is actively required for transposition, a phage lambda-like excision-integration system is not favoured. A number of different copy-replication models are available. Harshey and Bukhari (Cold Spring Harbor) have visualized the DNA structures found when the bacteriophage Mu is induced. Mu can only replicate by transposing so a large number of transposition events are found in a single cell. Harshey and Bukhari see DNA structures which can be explained by a 'roll-in' mechanism which can initiate at either end of Mu.

The gene conversion system which controls mating type in yeast may be similar to a transposable system but under careful cellular control. The cassette model for mating type expression holds that

information from either of two silent loci is sequentially expressed after transposition to a third (MAT) active locus this transposition is similar to that in prokaryotes as the information remains unchanged at the donor site. The focus of both molecular and genetic studies reported by three groups, Nasmyth (University of Washington), Klar (Cold Spring Harbor) and Haber (Brandeis University), is the cellular control of these three loci. It is not clear how sequences transposed to the MAT site become active. The active MAT site is a complex locus each MAT allele coding for two genes, divergently transcribed from opposite strands (Nasmyth and Klar). Yeast strains carrying recessive mutations in unlinked regulatory genes can express the silent mating type cassettes which shows that the silent copies, normally under negative control in trans, are relieved from this control when the information is switched to the active mating type locus. This rules out a system of the type demonstrated for sarcoma viruses where the cellular *sarc* structural gene and a strong promoter can be brought together to transform cells with high efficiency. Lambda-like independent excision and integration events were ruled out by evidence presented by Klar. He favored a duplicative transposition mechanism where the silent locus physically interacts with MAT since recombinants between MAT and the silent loci have been observed. The genetic studies of Haber (Brandeis University) were also consistent with this view.

Another eukaryotic system where genes are expressed in a mutually exclusive fashion occurs in the protozoan responsible for sleeping sickness. This parasite escapes from the host immune response by altering the dominant surface antigen which constitutes 95% of surface protein. Individuals of the species

Trypanosoma brucei have the ability to express one of many hundreds of special genes as this surface protein and so escape the host immune response. It appears now from work reported by Borst (University of Amsterdam) that at least some genes are moved to special genomic sites to become active. This may be the first generalization of the yeast cassette model and it is intriguing to speculate that other classes of genetic differentiation may be achieved by moving information around the genome.

What are we to make of all this genetic instability? Work on the proteins which mediate genetic recombination: *recA*, *recBC*, *synaptase* and the *topoisomerases*, emphasizes the ease with which the DNA may be rearranged. At least two conclusions follow: first, where genetic maps are conserved between bacteriophages or between the human and mouse X chromosomes this arrangement must have a selective advantage. Second, there may be systems where widespread genetic rearrangement is favoured over the usual conservative gene arrangement. We know of some of these situations; the recovery of bobbed *Drosophila* and the amplification of *Xenopus* ribosomal genes. A report by Jones (Edinburgh University) suggests that

we may no longer be able to relegate these rearrangements to the miscellaneous file. He has found a sequence in the heterogametic W chromosome of female snakes which is conserved in echinoderms, *Drosophila* and mammals. This repeated sequence has at least two dramatic properties. Its sequence arrangement in different tissues of *Drosophila* varies and in mammals, its arrangement in males and females differs. When sex reversed Sxr mice which are phenotypically male but have a female karyotype were tested they exhibited the male pattern. If it is true that Sxr males are not Y autosome translocations but express constitutively the gene normally triggered by a Y chromosome signal, the implication is that the genomic rearrangement is a secondary sexual characteristic. This hypothesis could be tested by examination of another mouse strain Tfm (testicular feminisation) and of bovine freemartins where hormonal regime rather than genetic inheritance is known to mediate altered sexual differentiation. It is a measure of how far our views have changed that Jones' findings were met with interest and not instant disbelief. Of course we are now confident that the clones and blots will tell all. □

The nature of gravitation

from A.H. Cook

EXPERIMENTAL studies of gravitation have been discouraged by the attitude of many theoreticians who, from the time of Newton, have taken gravitation as something 'given'. Experimenters are not, however, so easily put off by theoretical detachment even though the very small strength of gravitation makes its investigation difficult — the gravitational force between proton and proton is about 10^{-39} of the electrical force which, in practice, means that any laboratory study of gravitation will be bedevilled by all manner of extraneous disturbances.

A simple-minded approach to the experimental study of gravitation is to look at the inverse square law and ask such questions as: what is the 'mass'? Is it always proportional to inertial mass or are there differences from substance to substance? Is there any screening of one body by another, as with electrostatic forces? Is the 'constant' of gravitation constant, or does it depend on velocity, time, position, or the direction of the line joining two attracting bodies, and finally, is the inverse square law strictly obeyed?

We have experimental answers to these questions for the electrostatic force, and they are expressed in the appropriate Maxwell equations. We have some answers for gravitation. Experiments for many

years have failed to detect any dependence of gravitation upon material — all hadrons are equivalent gravitationally to within about 1 part in 10^{11} . A few astronomical and geophysical observations have shown the absence of any shielding effect to about the same limit. Geophysical observations of Earth tides show that if there is any dependence of gravitation on direction it cannot exceed 1 part in 10^8 . These and other effects can be incorporated in certain sorts of theory known as parameterised post-Newton (PPN) gravitation theories, provided sufficient parameters are included. If some of these parameters differ from the values predicted from general relativity, then certain effects in celestial mechanics would occur and might be detectable in refined observations of space craft or the Moon. Although such observations, for example, laser ranging measurements of the orbital motion of the Moon, are now possible, no derivations from the predictions of general relativity have so far been detected.

It should be emphasized that the type of experiment being considered is quite different from attempts to detect gravitational radiation. No one seriously doubts that radiation is entailed by general relativity but the question is whether any object radiates strongly enough to be observed with techniques available on Earth.

With the growth of interest in unified theories of gravitation and other inter-

100 years ago

The *Philadelphia Record* deserves credit for its successful efforts to break up the sale of bogus medical diplomas. These diplomas were chiefly sold abroad, and it is appalling to learn, that 11,000 of them have been issued during the past few years. "It was well known," the *Times* correspondent writes, "that Dr. John Buchanan, the Dean of 'The American University of Philadelphia,' and several other similar institutions, was engaged in this traffic; but as they were all properly chartered medical schools, and, though disreputable, existing under the sanction of law, the difficulty was to get evidence of the sale of diplomas. Diplomatic complaints about the traffic came from various Governments of Europe, and our people began to be restive under the stigma." By the clever tact of the city editor of the *Record*, however, Buchanan has been brought within reach of the law, and the detectives are on his track. He attempted to put them off the scent by getting a man looking like himself to pretend to drown himself; this bogus case of drowning, however, has deceived nobody.

From *Nature*, 22, 16 September, 465, 1880

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actions, it is natural to suppose that there should be a particle having a finite mass which arises from some broken symmetry in the field theoretic description of gravitation, and it has been suggested that the gravitational potential energy of two masses would not be simply proportional to $1/r$, where r is their separation, but would be given by

$$\frac{G_0 M_m}{r} (1 + \alpha \exp(-r/\lambda)).$$

This gives an inverse square law dependence at small distances ($r < \lambda$) and at large distances ($r > \lambda$) and the ratio of the respective "constants" of gravitation is $(1 + \alpha)$.

Could this, or some other departure from the inverse square law be detected? Long (*Nature* 260, 417; 1976) claimed to have found such an effect but in a recently published experiment by Spero *et al.* (*Phys. Rev. Lett.* 44, 1645, 1980) no deviation was detected. As is well known, very delicate tests of the inverse square law of electrostatics can be made using the principle that the field inside a charged sphere vanishes. Construction of a hollow sphere for a gravitational experiment is hardly possible so Spero and his colleagues investigated the gravitational field inside a long tube. Were the tube infinitely long there would be no net internal field but with a finite cylinder there are end effects. The experiment was designed to use a sensitive torsion balance to measure the change in force on a test mass in different radial positions within a long vertical

cylinder. A servo-control was applied to the torsion balance to maintain it in a constant position and the restoring force necessary to do so was recorded for different positions of the cylinder relative to a test mass suspended from one end of the balance beam. To attain a useful precision, the experiment had to be very carefully designed. Corrections were determined for the end effects and other stray gravitational fields and precautions were taken against magnetic and convective forces. The experiment depended on the material of the cylinder (which was of stainless steel, 60 cm long, of 6 cm internal diameter and a mass of about 10 kg) being very uniform, and a careful study of the cylinder had therefore to be undertaken. The measured torques were of the order of 5.10^{-13} Nm, showing the extreme delicacy that must be attained in worthwhile experiments on gravitation. The observations agreed with the torque predicted for pure inverse square law attractions to 2.10^{-15} Nm, a result that is inconsistent with Long's earlier study.

It can be said that no deviation of the behaviour of gravitation from the predictions of general relativity has as yet been established experimentally, and one may suspect that none will be. That, however, is not a reason for abandoning experiments such as those mentioned earlier. Gravitation is such a fundamental interaction that it is a worthwhile aim to establish its properties to the very highest precision, particularly since to do so presents a daunting but irresistible challenge to the experimenter. □

systematically with increasing geological age — but do allow a better contouring of the global data set.

One issue beginning to be resolved by the increasing number of heat flow observations is the nature and width of heat flow transitions. That heat flow transitions of 30 mW m⁻² and greater commonly occur over lateral distances of 10 to 50 km was demonstrated in an analysis of existing data for rift valleys (Morgan, New Mexico State University), by new data from the Southern Rocky Mountains (Decker, University of Wyoming), and by independent new surveys by Stone (University of Arizona) and Chapman and colleagues (University of Utah) of the transition from the Basin and Range to the Colorado plateau physiographic province. In areas where few measurements existed, the transition had been considered to be broad and the source thermal perturbation confined to the asthenosphere. The observations of sharp transitions imply that heat sources must be shallow, within the lithosphere or possibly even within the crust. As the calculation of geotherms, regularly used to predict rheology, mineralogy and partial melting in the lower lithosphere, is critically dependent on the depth distribution of heat sources, modification of much used purely conductive geotherms in certain regions now seems appropriate (as suggested by Lachenbruch & Sass *Geol. Soc. Amer. Memoir* 152, 209; 1978).

A further caution to those who seek deeper meaning from all heat flow anomalies came from three studies of the role of groundwater in redistributing heat. Heat flow investigators for several years have inferred the role of groundwater in transferring heat, especially in very young oceanic crust, but the direct demonstration of those flows has been difficult. Therefore, the experiments described by D. Abbot and colleagues (Lamont-Doherty) on the simultaneous measurement of excess pore pressure and temperature gradients in sediments covering 22 Myr old crust in the Guatemala Basin is of special significance. The objective was to explain very low observed heat flows of 1 to 20 mW m⁻² in the region instead of the 105 mW m⁻² expected on the basis of crustal age. Although measured temperature-depth profiles were linear, usually an indication that convective disturbances are absent, all of the new measurements yielded negative pore pressures within the sediment, indicating downward fluid velocities between 10^{-6} and 10^{-7} cm s⁻¹. Such velocities are sufficient to decrease heat flow by a factor of 10. More important, they confirm the long held suspicion that many subnormal oceanic heat flow areas actually delineate the downgoing side of a sea floor convec-

Terrestrial heat flux

from David S. Chapman

PRESENT day heat loss through the Earth's crust is approximately 4×10^{13} Joules and as such is of major importance in geodynamic processes. The source of the heat, its mode of transport and its geographical pattern of variation are still questions which remain largely unanswered and they provided the central theme for a recent conference*.

Heat flow measurements have still not been made from many parts of the world so one of the highlights of the conference was the addition of data from China to the global heat flow map. Ji-Yang Wang (Academia Sinica, Beijing) reported that twenty-two values from north China fall into the following patterns: heat flow of 32 mW m⁻² in the Archean crust of central Yanshan; 54 to 75 mW m⁻² in the neotectonic Huailai and Yanquing basins, and 61 to 84 mW m⁻² on the block faulted North China Plain (see

J. Volcanol. Geotherm. Res. in the press) where tectonic activity, according to Wang, "has been strengthening since the Mesozoic era". The Chinese territory also received attention from Anderson (Lamont-Doherty Geological Institute, Columbia) in his report of the first detailed heat flow profile across a continental margin. The demonstration of stable bottom water temperatures on the margin, a prerequisite for shallow marine measurements, will encourage further experiments in spite of the monotony of the results — a uniform 90 mW m⁻² with one unexplained anomaly in 88 measurements.

In another pioneering effort, Bucher and Decker (University of Wyoming) reported values between 50 and 84 mW m⁻² in the Ross Island dry valley area of Antarctica. These results significantly extend the coverage of heat flow measurements on the globe. None of the new results depart from the heat flow patterns established elsewhere in the world — heat flow is elevated in young orogenic provinces and decays

*A Spring American Geophysical Union meeting titled "Heat flow and crustal properties" was held in Toronto from May 22 to May 27, 1980.

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tion system.

On a larger scale of tens of kilometers, groundwater flow was also shown to have major effects on observed heat flow patterns. Lohse, Morgan and Swanberg (New Mexico State University) reported 60 new heat flow determinations from the Rio Grande Rift zone and interpreted the highly variable pattern in terms of ascending warm water in rift fracture zones and intrabasin groundwater flow in the Rio Grande River drainage system. In perhaps the best example of redistribution of crustal heat over lateral distances of hundreds of kilometers, Majorowicz (Warsaw University) demonstrated that a relatively low heat flow of 50–75 mW m⁻² in the Rocky Mountain foothills of Alberta and an abnormal heat flow of 70–100 mW m⁻² in the northern Prairies Basin could only be explained by regional groundwater flow.

In contrast to the previous discussions on patterns and mode of transport of the terrestrial heat flux, two papers addressed the source of heat, at least the crustal radiogenic portion now thought to account for

about 40% of the continental flux. Our present understanding of this radiogenic heat distribution hinges on the empirical relationship between surface heat flow and surface radioactive heat production first demonstrated in 1968 for plutons by Birch and co-workers, and modeled soon afterwards by Lachenbruch. According to these early models, plutons undergo an upward enrichment in uranium, thorium and potassium, the important heat producing elements. Their combined depth distribution is characterized by the length parameter *b*, the slope of heat flow-heat production line for that pluton. Any of three depth distributions, constant to depth *b*, linearly decreasing to depth 2*b*, or exponentially decreasing with characteristic decrement *b*, satisfy the heat flow-heat production relationship, although the latter makes the most geological sense.

Now Gosnold (Nebraska University) and Jaupart (University of Paris) have independently suggested modifications to this model and its interpretation. Gosnold has combined fission track, petrographic and oxygen isotope studies on samples

from several plutons within the Idaho Batholith. His results suggest, not surprisingly, that heat generation is largely controlled by water-rock interaction in the upper 10 km of the crust. In such a case the overall vertical heat production distribution could be exponential throughout the crust but an inflection in the curve would occur at the lowest depth of meteoric water interaction. Jaupart's modification is more severe. He questioned the assumption that uranium, thorium and potassium have identical depth distributions. He has inferred from his preliminary analysis, although based on surface sampling only, that the three elements have different depth distributions but are similar from one geological province to another. Both these suggestions have interesting geochemical and geophysical implications and it is a pity therefore that the issues may not easily be resolved. The characteristic scale for these processes and the radioactive enrichment is 10 km, and few, if any, geological locations permit sampling over that vertical extent in the igneous crust. □

Myosin phosphorylation

from Dixie W. Frederiksen

REGULATION of muscle contraction and cell motility occurs by the control of the interaction of actin and myosin. Primary control is exercised by the intracellular calcium concentration and is modulated at secondary levels by various mechanisms that seem to depend upon the source of the tissue or proteins examined. Myosin phosphorylation plays an important role both in the primary regulation and in the secondary modulation of contraction and motility¹.

In vertebrate striated muscle the binding of calcium to troponin, a protein on the actin filament of the muscle sarcomere, is clearly the event that triggers actin-activation of the MgATPase. Although the direct steric blocking model for this actin-linked regulatory mechanism² has been challenged recently³ there is little debate that troponin and tropomyosin are responsible for primary regulation in vertebrate striated muscles. At the secondary level, phosphorylation of a troponin subunit by cyclic AMP-dependent protein kinase has been shown to result in decreased actin-activated MgATPase activity of striated muscle myosin⁴. Furthermore, although striated muscle myosin phosphorylation was discovered sometime ago by S. V. Perry and his colleagues at the University of Birmingham⁵, the function of this modification is still largely unknown. The phosphorylation is presumed to play a

secondary role in modulation of the contractile process.

In many smooth muscle and nonmuscle actomyosin systems it seems clear that the calcium-sensitive mechanism responsible for primary regulation is myosin-linked through phosphorylation of one of the small subunits of the myosin molecule. David J. Hartshorne and his collaborators⁶ at the University of Arizona and Robert S. Adelstein with his colleagues at the N I H⁷ have shown in several elegant experiments that the binding of a calcium-calmodulin complex to myosin light chain kinase and the subsequent phosphorylation of myosin are the initial steps in actin-activation of smooth muscle and some of the nonmuscle myosin MgATPases. The secondary modulation of contraction/motility in these systems seems to include one or more different processes. Cyclic AMP-dependent protein kinase-catalysed

phosphorylation of myosin light chain kinase was discovered by the Adelstein group⁸ and found to result in decreased activity of the myosin light chain kinase. In addition, direct interaction of calcium with phosphorylated smooth muscle myosin has been reported to modulate the actin-activated MgATPase⁹.

The effects of myosin phosphorylation on the contractile apparatus are complex and subtle and reports of the influence of myosin phosphorylation on striated muscle proteins *in vitro* have not been forthcoming. As a result, investigators have turned to the examination of intact muscle for evidence of the effects of phosphorylation. Michael Bárány and his colleagues at the University of Illinois Medical Center¹⁰ have used frog muscle and James T. Stull and his group at the University of Texas Health Science Center¹¹ have used mammalian striated muscle to monitor the phosphorylation. In these *in situ* studies, phosphorylation of myosin light chain was observed to be correlated with the stimulation of the muscle. Bárány has suggested that the function of the phosphorylation may be to increase the actin-myosin affinity by the formation of a salt-bridge between the phosphoryl group on myosin and the actin-bound calcium on the thin filament. Such an increased affinity would lead to an increased rate of combination of myosin crossbridges with the actin filament.

The phosphorylated myosins of smooth muscle and several nonmuscle systems, unlike their striated muscle counterparts,

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Bishop Wilberforce: Natural Selection and the Descent of Man

from Richard W. Wrangham

Samuel Wilberforce, Bishop of Oxford, was the man who played into T.H. Huxley's hands at the British Association's meeting in 1860. They were debating the merits of Darwinism, and the atmosphere in the crowded room was highly charged. Wilberforce was a fine speaker — "Like an olive, his nature secretes his own oil", wrote *Punch*¹ — and he ended his well-prepared attack on evolutionary theory by asking if his opponent were prepared to admit apes to his own ancestry. The reply was deadly. An ape, said Huxley, would be a preferable grandfather to an intelligent man who used his skills to bring ridicule to

science.² It won the audience and silenced the Bishop, who never spoke again on evolution.

Huxley went on to build a career out of promoting Darwinism, while Wilberforce returned to ecclesiastical matters. But how did he come to feel about evolution? His only published opinion was a review of the *Origin of Species*, written before the debate with Huxley.³ There he denied unambiguously that evolution was tenable, though he approved of natural selection. He understood the arguments well and used them to maintain that selection acted to keep species constant, not to change them⁴.

Now a clue to his subsequent feelings has emerged. The poem below was found earlier this year in his private papers, and is undated. Its theme, "degradation by natural selection", is from Richard Owen's review of the *Origin*.⁵ Wilberforce saw all too well the clash between evolutionary theory and his religious beliefs, and in 1860 he had come out in attack. But in this poem he side-stepped the conflict and retreated to comedy. It implies a man too committed to accept the evolutionary argument, yet too honest in the end to deny it. Who knows? Darwin may have had one more convert than he knew.

"Lines written on hearing that Professor Huxley had said that 'he did not care whether his grand-father was an Ape'."

Oft had I heard, but deemed the tale untrue,
That man was cousin to the Kangaroo;
That he before whose face all nature quailed,
Was but the monkey's heir, though untailed;
And that the limber Ape, whose knavish ways
And tricks fantastic oft our laughter raise,
Was just what we were in some previous state,
Ages ere Noah shipped his living freight.
But now a learn'd Professor, grave and wise,
Stoutly maintains what I supposed were lies;
And, while each listening sage in wonder gapes,
Claims a proud lineage of ancestral Apes.
Alas! cried I, if such the sage's dreams,
Save me, ye powers, from these unhallowed themes;

From self-degrading science keep me free,
And from the pride that apes humility!
But O should fate bring back these dreams accursed,
And shuddering Nature find her laws reversed;
Should this, the age of wonders, see again
Men sunk to monkeys, monkeys raised to men:
Be mine the lot, on some far-distant shore,
Where Science wearies not nor savants bore —
Where no learn'd Apes our fallen race may scorn,
Nor point the moral which our tails adorn —
To shun the sight of metamorphosed friends,
Till time again shall shape their altered ends,
To soothe each fond regret, howe'er I can;
And, at the least, to dream myself a Man!

S. Wilberforce

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have properties that are readily observed *in vitro* and are clearly distinct from those of the unphosphorylated protein. The influence of phosphorylation on the potentiation of the actin-activated myosin MgATPase has been the major focus of attention. Upon phosphorylation of the myosin light chain, the MgATPase activity of the protein in the presence of actin is increased ten- to twentyfold¹².

A third manifestation of the effect of myosin phosphorylation has now been observed *in vitro* with myosin from smooth muscle and nonmuscle cells. Working with myosins from thymus, platelets and gizzard, John Kendrick-Jones *et al.* show in this issue of *Nature* (p.233) that bipolar thick filaments are formed under physiological conditions only if the myosins are phosphorylated. These synthetic thick filaments are stable in the presence of millimolar concentrations of MgATP. Shorter filaments are formed of un-

phosphorylated myosins *in vitro* at low ionic strength, but these are completely dissociated well below physiological concentrations of MgATP. These results are a striking extension and confirmation of the work by Hiroshi Suzuki and his collaborators at the Tokyo Institute of Technology¹³ who first observed that the state of gizzard myosin phosphorylation has a profound effect upon the ability of the protein to form extended aggregates.

The observations of Suzuki and of Kendrick-Jones are particularly important because they resolve some puzzles and raise some interesting questions. If smooth muscle and non-muscle myosin must be phosphorylated in order to form thick filaments *in vivo*, then the absence of such filaments in many electron micrographs of smooth muscle and nonmuscle cells might be explained by the absence of phosphorylated myosin. Furthermore, another possible function of phosphorylation in

striated muscle becomes clear — phosphorylated, striated muscle myosin may constitute thick filaments more stable and/or more ordered than the unphosphorylated myosin.

The questions are intriguing. How does filament formation contribute to the observed actin-activation of the smooth muscle and nonmuscle MgATPase? Is actin-activation due to an increased order in the myosin array and, therefore, to an increased accessibility of myosin to actin? Another very important question raised by Kendrick-Jones has to do with how modification of the head portion of myosin might influence the association and packing of the opposite, tail portion of the molecule. No doubt subsequent studies by these and other investigators will address these problems and will shed even more light upon the nature of the finely tuned control in the contractile protein complex.

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ARTICLES

Expression of human fibroblast interferon gene in *Escherichia coli*

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The human fibroblast interferon gene was inserted in a thermoinducible expression plasmid under control of the phage lambda P_L promoter. The primary translation products predicted on the basis of the plasmid constructions were hybrid proteins starting with β -lactamase or phage MS2 polymerase information followed by the total preinterferon. On induction, antiviral activity, whose physico-chemical, immunological and biological characteristics closely corresponded to those of authentic human fibroblast interferon, was synthesized. Processing to a size compatible with mature but unglycosylated authentic product was observed.

ON exposure to viruses or other specific inducers most vertebrate cells secrete protein(s) with broad antiviral action known as interferons. Human interferons are being intensively studied for their antiviral¹, anticellular² and immunomodulating³ activities. Clinical trials have been carried out mainly with leukocyte interferon but also with fibroblast interferon, and some promising results have been obtained with both types in the treatment of viral diseases and cancer^{1,4-9}. Tests with fibroblast interferon have been severely restricted by its very limited availability.

In a previous report¹⁰, we described the construction and characterization of chimaeric plasmids containing human fibroblast interferon (HF-IF) cDNA. Two other groups have constructed plasmids containing either human leukocyte¹¹ or human fibroblast¹² interferon cDNA and in the former case, interferon-related polypeptides, as judged by biological and immunological criteria, were detected in *E. coli* strains harbouring the chimaeric plasmids. We have now inserted the HF-IF coding sequence derived from our original clones into appropriate sites on specifically constructed expression vehicles which contain the strong leftward promoter (P_L) of bacteriophage λ . The functioning of the promoter could be controlled by using host strains which synthesize a temperature-sensitive repressor (cI-ts). We describe here how plasmids containing P_L in front of the HF-IF coding sequence direct the synthesis of polypeptides with human fibroblast interferon activity in *E. coli*.

Construction of plasmids allowing expression of HF-IF

Construction of the different plasmids containing HF-IF DNA under the control of lambda P_L is schematically represented in Fig. 1. The formation and use of acceptor plasmids pPLa2311, pPLa8 and pPLc24 will be published in detail elsewhere (E. Remaut *et al.*, in preparation).

None of the chimaeric plasmids previously described contains an uninterrupted HF-IF gene¹⁰. A complete and continuous coding sequence for HF-IF was reconstituted by inserting an *EcoRI*-*PstI* fragment from pHFIF-6 and a *PstI*-*HaeII* fragment from pHFIF-7 into the plasmid pPLa2311. From the resulting plasmid, designated pPLa-HFIF-67-1, a *BglII* fragment was

excised and ligated into the *BamHI* site of the β -lactamase region of pPLa8 in the sense orientation with respect to the P_L promoter. The known nucleotide sequence around the *BamHI*-*BglII* junction in this plasmid, pPLa-HFIF-67-12, predicts that a polypeptide initiated at the AUG of the β -lactamase part will terminate on a double amber stop codon in the 5'-untranslated region of the HF-IF gene, 23 nucleotides before the HF-IF initiating AUG (data not shown).

pPLa-HFIF-67-12 Δ 19 was derived from pPLa-HFIF-67-12 by deleting a *HindIII* fragment starting within the β -lactamase gene and extending up to three nucleotides before the HF-IF initiating AUG (Fig. 1). From the known nucleotide sequence of the β -lactamase gene (as determined on the progenitor pBR322)¹³ and of the HF-IF gene¹⁰, a continuous reading frame starting at the initiating AUG of the β -lactamase gene and running up to the terminating UGA of the HF-IF gene is predicted. The expected fusion polypeptide consists of 82 amino acid residues of the β -lactamase protein, one amino acid coded for at the fused *HindIII* site, and the complete polypeptide (including the putative signal sequence) specified by the HF-IF gene. The predicted sequence around the junction is: β -lactamase gene moiety-GUU.AAC. AUG-HFIF gene, where the GUU triplet codes for amino acid 82 of the β -lactamase protein.

Alternatively, a hybrid plasmid with the controllable lambda P_L promoter in the clockwise orientation was constructed. The acceptor plasmid was pPLc24, which contains the P_L promoter followed by an *EcoRI*-*BamHI* fragment (derived from pMS2-7 [ref. 14]) containing the ribosome binding site and part of the MS2 polymerase gene. The pPLa-HFIF-67-1 *BglII* fragment containing the HF-IF gene was inserted into the *BamHI* site of pPLc24, resulting in loss of *BamHI* and *BglII* sensitivity but formation of *Sau3AI* sites at the joints (Fig. 1). In this new plasmid, pPLc-HFIF-67-8, a continuous reading frame starting at the initiating AUG of the MS2 polymerase gene and terminating at the UGA of the HF-IF gene, can be predicted on the basis of the known nucleotide sequences of the MS2 polymerase gene¹⁵ and of pHFIF-6 and pHFIF-7 (ref. 10). The expected fusion protein consists of the N-terminal 98 amino acids of the MS2 polymerase moiety, 27 amino acids coded for by sequences between the *BglII* site and the initiating AUG of the HF-IF gene, followed by the complete HF-IF coding region,

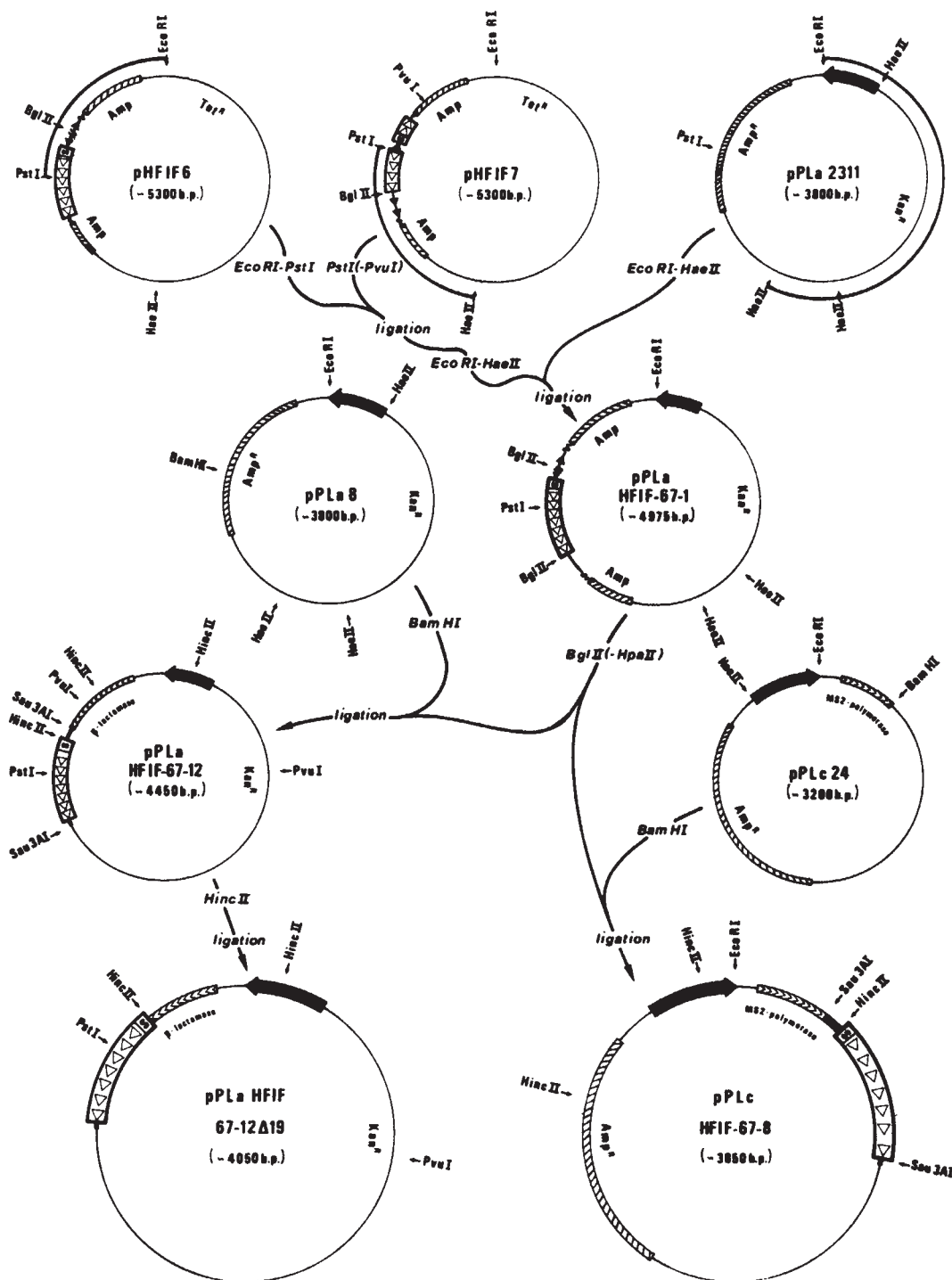


Fig. 1 Schematic outline of the construction of the different plasmids. The HF-IF DNA is indicated by a heavy line, the boxed area being the coding region with the putative signal peptide (S); the triangles indicate the 5' → 3' direction of the insert. The heavy arrow indicates the λ -promoter control element. The ampicillin-resistance region is shown as a shaded area. An interruption of the base line in the insert shows the cross-over region of the inversions of our original HF-IF cDNA-containing plasmids¹⁰. dA-dT tails are indicated by a wavy line. Amp, β -lactamase region coding for resistance to ampicillin and carbenicillin; Kan, region coding for kanamycin resistance. To construct pPLa-HFIF-67-1, pHFIF-6 DNA was cleaved with *Eco*RI and *Pst*I and ligated to pHFIF-7 which had been cleaved with *Pst*I and *Pvu*I. Following ligation, the mixture was digested with *Eco*RI and *Hae*II and ligated to pPLa2311 which had been digested with *Eco*RI and partially with *Hae*II. The segments of the outer circles indicate the fragments retained in the pPLa-HFIF-67-1 construction: only those restriction sites relevant to the constructions are indicated. Transformants were obtained in C600r⁺m⁺ (λ) with selection for kanamycin resistance and screened for sensitivity to carbenicillin. The structure of a representative plasmid, pPLa-HFIF-67-1, was confirmed by digestions with *Eco*RI, *Pst*I, *Bgl*II and *Hinc*II (= *Hind*II). To construct pPLa-HFIF-67-12, the HF-IF gene was excised from pPLa-HFIF-67-1 with *Bgl*II, followed by fragmentation of the remaining part of the plasmid with *Hpa*II, which does not cut the HF-IF gene. The DNA was ligated to pPLa8, which had been opened with *Bam*HI. After ligation, the mixture was redigested with *Bam*HI to eliminate recircularized pPLa8 acceptor plasmids. Transformants were obtained in C600r⁺m⁺ (λ) by selection for kanamycin resistance. The structure of pPLa-HFIF-67-12 was examined by analysis with *Pst*I and *Hinc*II. To construct pPLa-HFIF-67-12Δ19, pPLa-HFIF-67-12 was partially digested with *Hinc*II and ligated at a low DNA concentration (0.01 μg ml⁻¹). The mixture was then digested with *Xho*II, an isoschizomer of *Pvu*I producing 3'-protruding ends²⁶, and religated at low DNA concentration, thereby substantially reducing the probability of religation of the original pPLa-HFIF-67-12 plasmids. The structure of pPLa-HFIF-67-12Δ19 was examined by digestion with *Hinc*II and *Pvu*I. To construct pPLc-HFIF-67-8, the DNA of pPLa-HFIF-67-1 was digested with *Bgl*II and ligated into the *Bam*HI-opened pPLc-24 DNA. The ligation mixture was redigested with *Bam*HI to eliminate religated parental pPLc-24 plasmids. Transformants were selected for resistance to carbenicillin. The insertion and orientation of the *Bgl*II HF-IF fragment was established by *Hinc*II digestion. Restriction enzymes (Biolabs, Boehringer or BRL) were used according to the manufacturer's specifications. Ligations were carried out at 22 °C with T4 DNA ligase in Tris-Cl (50 mM, pH 7.6), MgCl₂ (5 mM), β-mercaptoethanol (7 mM), ATP (50 μM), (ref. 27). All plasmids were subsequently transferred into *E. coli* M5219 (K12 M72 lac_{am} trp_{am} Sm^R [AC1857ΔHI bio 2521])¹⁶. Enzymatic reactions were stopped by heating at 65 °C for 10 min.

including the signal peptide. The predicted sequence around the junction is

-Trp-Asp-Leu-Gln-Phe-Arg-Arg-Gln-Pro-
MS2 polymerase gene moiety-UGG.GAU.CUU.CAG.UUU.CGG.AGG.CAA.CCU.

Phe-Glu-Ala-Phe-Ala-Leu-Ala-Gln-Gln-Val-Val-Gly-Asp-Thr-Val-Arg-
UUC.GAA.GCC.UUU.GCU.CUG.GCA.CAA.CAG.GUA.GUA.GGC.GAC.ACU.GUU.CGU.

Val-Val-Asn-Met-
GUU.GUC.AAC.AUG-HFIF coding region

The first amino acid, tryptophan, corresponds to position 98 of the MS2 polymerase.

All constructed chimaeric plasmids were transformed into *E. coli* C600r λ and, after characterization, transferred into *E. coli* M5219 (ref. 16), allowing the temperature-dependent controlled expression of the lambda P_L (E. Remaut *et al.*, in preparation).

Detection of IF activity in bacterial extracts

Transcription from the P_L promoter on the plasmids can be turned on by shifting the growing culture from 28 to 42 °C. The synthesis of IF-related product(s) was examined by assaying an S100 extract of the bacteria for antiviral activity (Table 1). The cells were lysed either by lysozyme treatment followed by freeze-thawing or by heating in 1% SDS, 1% β -mercaptoethanol, 5 M urea. The extracts of temperature-induced *E. coli* M5219 containing pPLa-HFIF-67-12 Δ 19 or pPLc-HFIF-67-8 showed a clear antiviral activity, which was reproducibly higher with the latter plasmid. The same non-induced strains as well as induced M5219 containing a reference plasmid (pPLa8) did not show any detectable activity. In M5219 containing pPLa-HFIF-67-12, trace amounts of antiviral activity were occasionally detected after temperature shift (data not shown), presumably due to a rare reinitiation event. The much higher activity obtained after lysis with the SDS, β -mercaptoethanol, urea mixture indicates a possible nonspecific sticking of the antiviral product(s) to bacterial components, for example, cell membranes or nucleic acids. In parallel experiments in which authentic HF-IF was added to a control bacterial extract obtained by lysozyme treatment, only 10–40% of the activity was recovered.

Low but significant amounts of antiviral activity were detected in the supernatant after osmotic shock of induced M5219 transformed with pPLc-HFIF-67-8 (Table 1). When a more severe method of periplasmic extraction was used (that is, spheroplast formation), some activity was also detected with induced M5219 transformed by pPLa-HFIF-67-12 Δ 19. These results suggest that at least some of the bacterial HF-IF may be secreted into the periplasmic compartment, perhaps concomitantly with the

removal of the signal peptide; other explanations, however, cannot be excluded.

Characterization of the bacterial IF activity

The antiviral activity detected in the above-mentioned extracts of induced bacteria was tested for several biological and physical properties characteristic of HF-IF (Table 2). First, the antiviral activity was non-dialysable; after dialysis for 16 h at neutral pH the antiviral activity was retained, albeit often at reduced levels (which was also the case for authentic HF-IF preparations). The observed decrease is presumably due to nonspecific sticking to the dialysis membranes, as HF-IF is known to be rather hydrophobic¹⁷, and the unglycosylated bacterial form may be even more so. The antiviral activity could be recovered after precipitation with 67% saturated ammonium sulphate, a concentration known to precipitate HF-IF¹⁸.

When tested for stability at pH 2, a common property of fibroblast and leukocyte interferon¹, bacterial HF-IF proved to remain active (Table 2), although again there was often partial loss of activity, but this was also the case with reconstituted authentic HF-IF controls.

The sensitivity of the bacterial HF-IF activity to protease was tested by treating the diluted bacterial extracts with increasing amounts of trypsin. The activity was abolished at the same concentration of trypsin that abolished the activity of authentic HF-IF added to an inactive control lysate.

HF-IF, in contrast to leukocyte interferon, is stable after heating in 1% SDS, 1% β -mercaptoethanol, 5 M urea¹⁹, although we only obtained 10–20% recovery of activity with authentic HF-IF, either alone or in the presence of an inactive bacterial extract (data not shown). The bacterial HF-IF activity remained active in these conditions, as lysis of induced bacteria in this solution resulted in extracts with the highest antiviral titre (Table 1).

The antigenic properties of the *E. coli* IF activity were compared with those of authentic HF-IF. Serial dilutions of goat anti-HF-IF antiserum were incubated with diluted extracts containing bacterial HF-IF activity and with control HF-IF preparations in the presence or absence of an inactive bacterial lysate. The bacterial IF activity was neutralized by the specific antiserum, but some differences were noted in the neutralizing antibody titres for bacterial IF and authentic HF-IF (Table 2). Small differences in neutralization titre were also reported for bacterial leukocyte IF when compared with authentic leukocyte IF¹¹. This can be explained by a difference either in antigenicity or in specific IF activity of these bacterial proteins relative to authentic IF.

Table 1 Interferon activity in extracts of *E. coli* M5219 transformed by expression plasmids containing the HF-IF coding sequence

Plasmid	Temperature	Interferon activity (units per ml extract) in			
		S100 extracts after lysis by lysozyme and freeze-thawing (I)	S100 extracts after lysis with SDS, β -mercaptoethanol, urea (II)	Periplasmic fraction: osmotic shock (III)	Periplasmic fraction: spheroplast formation (IV)
pPLa-HFIF-67-12 Δ 19	28 °C	<3; <2	<30; <100	<2	2
	42 °C	200; 20	200; 2000	<2	10
pPLc-HFIF-67-8	28 °C	<3; <2	<100; <100	<2	<2
	42 °C	200; 50	2,000; 3,000	30	30
pPLa8	42 °C	<3; <2	<100; <100	<2	<2

LB medium (150 ml) was inoculated with 1/500 volume of a fresh seed culture, saturated at 28 °C, and maintained with vigorous shaking at 28 °C until a cell concentration of 2×10^8 ml⁻¹ was reached. Induction was by shifting the temperature to 42 °C and incubation of the cultures for 3 h to a final concentration of $4\text{--}6 \times 10^8$ cells ml⁻¹. The cells were collected and washed with Tris-HCl (50 mM, pH 7.4), NaCl (30 mM) and repelleted. Several different extraction procedures were used: I, the bacterial pellet was resuspended in a final volume (4 ml) with HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM) 3% calf serum, β -mercaptoethanol (3 mM), to which lysozyme (Sigma) was added to 1 mg ml⁻¹. After incubation at 0 °C for 30 min, the suspension was subjected to one or two freeze-thawing cycles. The S100 fraction was prepared by ultracentrifugation at 60,000 r.p.m. for 1 h in a Beckman SW60 Ti rotor. II, The cells were resuspended as in I and lysed in HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM), 3% calf serum, 1% SDS, 1% β -mercaptoethanol, urea (5 M) at 90 °C for 1–2 min. Clearing by ultracentrifugation was as in I. III, Osmotic shock procedure²¹: the bacterial cell pellet was resuspended in 20% sucrose, EDTA (100 mM), Tris-HCl (100 mM, pH 7.4), to a cell concentration of 1×10^{10} ml⁻¹. After 10 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The pellet was resuspended in water to a cell concentration of 1×10^{10} ml⁻¹. After 10 min on ice, the suspension was again cleared for 10 min at 10,000 r.p.m. To this osmotic shock supernatant was added HEPES-NaOH (50 mM, pH 7.0), NaCl (30 mM), β -mercaptoethanol (3 mM) and 3% calf serum. IV, Cells were resuspended in 3.6 ml of 0.1 M Tris-HCl, pH 8.0, 20% sucrose, to which 0.4 ml of lysozyme (5 mg ml⁻¹ in EDTA [20 mM]) was added. After incubation for 30 min at 0 °C, the suspension was centrifuged for 10 min at 10,000 r.p.m. The supernatant was adjusted to 3% calf serum. IF activity was measured by a cytopathic effect (CPE)-inhibition assay on human fibroblasts trisomic for chromosome 21 in microtitre trays. The cells were challenged with vesicular stomatitis virus (Indiana strain) and the CPE was recorded at 24 h. All assays included an internal HF-IF reference which was calibrated against the NIH HF-IF reference G023-902-527. The limit of detection, normally 1 unit ml⁻¹, was often elevated due to toxicity of certain samples (for example, for the samples obtained with method II, the limit of detection was 30–100 units ml⁻¹). The titres are expressed in units ml⁻¹, although it should be noted that they were obtained by 0.5 log₁₀ dilutions.

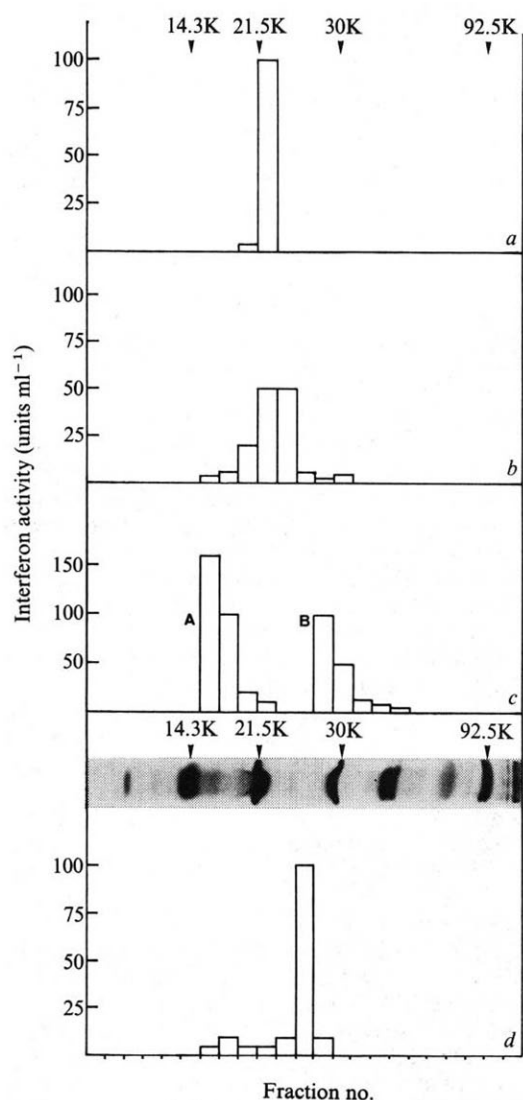


Fig. 2 Polyacrylamide gel patterns of bacterial HF-IF and authentic HF-IF. The following 100- μ l samples were loaded on slots of 2 cm width: *a*, authentic HF-IF in Eagle's minimal essential medium, 10% calf serum; *b*, authentic HF-IF in control bacterial extract of M5219/pPLA8 (42 °C); *c*, bacterial extract of M5219/pPLC-HFIF-67-8 (42 °C); *d*, bacterial extract of M5219/pPLA-HFIF-67-12 Δ 19 (42 °C). 14 C-labelled protein markers electrophoresed in an equivalent amount of bacterial extract of M5219/pPLC-HFIF-67-8 (42 °C) are shown as an insertion in *d*. Bacterial extracts were prepared by treatment with SDS, β -mercaptoethanol, and urea (compare with procedure II in legend to Table 1). All samples were boiled for 1 min before electrophoresis, which was run according to Laemmli²⁸ in 12.5% polyacrylamide gel. IF activity profiles (*a*, *b*, *c*, *d*) were obtained after elution of successive 0.5 cm gel slices for 15 h in 500 μ l of 0.5% bovine serum albumin in Tris (0.0125 M), glycine (0.096 M), 0.05% SDS followed by the antiviral IF assay (compare with legend to Table 1). Arrows with molecular weight corresponding to 14 C-labelled markers are indicated on top.

HF-IF is largely species specific, exhibiting little (if any) antiviral effect on heterologous cells¹. Consistent with this property, bacterial IF showed no detectable antiviral protection on cells of monkey, feline, rabbit or mouse origin (Table 3). With respect to the feline cells, bacterial and authentic HF-IF behave differently from human leukocyte interferon.

Further evidence substantiating the presence of active HF-IF in induced *E. coli* extracts was provided by demonstrating the induction of 2', 5'-oligoadenylate (2-5A) synthetase. Kerr *et al.*²⁰ first reported that interferon increases the level of this enzyme in susceptible cells. As shown in Table 4, appropriate bacterial extracts were able to enhance the incorporation of [α - 32 P]ATP in ppp5'A2'p5'A2'p5'A. The 2-5A synthetase-inducing activity of the bacterial extracts was proportional to

their antiviral activity. This bacterial IF activity was likewise neutralized by anti-HF-IF antiserum.

Size estimations of bacterial HF-IF

To estimate the molecular weight of the bacterial IF activity in comparison with authentic HF-IF, bacterial extracts were fractionated by polyacrylamide gel electrophoresis in denaturing conditions and the antiviral activity determined for eluates from successive gel slices (Fig. 2). Whilst authentic HF-IF showed a single peak of activity after electrophoresis, the bacterial IF activity appeared in two different peaks. Very accurate molecular weights could not be assigned because of insufficient resolution of the gel; this was mainly due to an overloading effect

Table 2 Characterization of the bacterial HF-IF activity

	Interferon titre (units ml ⁻¹)	
<i>a</i> , Dialysis at neutral pH:	Before	After
M5219/pPLC-HFIF-67-8 (42 °C) (I)	200	200
(II)	1,000	200
(III)	30	20
M5219/pPLA-HFIF-67-12 Δ 19 (42 °C) (I)	200	20
Control HF-IF in M5219/pPLA8 (42 °C) (I)	200	100
	200	10
<i>b</i> , Precipitation with (NH ₄) ₂ SO ₄ at 67% saturation:		
M5219/pPLC-HFIF-67-8 (42 °C) (I)	100	100
	100	200
M5219/pPLA-HFIF-67-12 Δ 19 (42 °C) (I)	100	100
Control HF-IF in M5219/pPLA8 (42 °C) (I)	20	20
	30	20
<i>c</i> , pH 2 treatment:		
M5219/pPLC-HFIF-67-8 (42 °C) (I)	100	20
(II)	5	5
M5219/pPLA-HFIF-67-12 Δ 19 (42 °C) (I)	100	10
Control HF-IF in M5219/pPLA8 (42 °C) (I)	1,000	100
<i>d</i> , Heat treatment in 1% SDS, 1% β -mercaptoethanol, 5 M urea (see Table 1)		
	Inactivating end point concentration (mg ml ⁻¹)	
<i>e</i> , Trypsin digestion:		
M5219/pPLA-HFIF-67-12 Δ 19 (42 °C) (II)	0.03	
M5219/pPLC-HFIF-67-8 (42 °C) (II)	0.03	
(1,000 units ml ⁻¹)		
Control HF-IF in M5219/pPLA8 (42 °C) (II)	0.03	
(1,000 units ml ⁻¹)		
M5219/pPLC-HFIF-67-8 (42 °C) (III)	0.03	
(30 units ml ⁻¹)		
Control HF-IF in M5219/pPLA8 (42 °C) (III)	0.03	
(30 units ml ⁻¹)		
<i>f</i> , Neutralization by antiserum		Neutralization titre (units ml ⁻¹)
pPLC-HFIF-67-8 (42 °C) (I)		10 ^{5.2}
(II)		10 ^{5.0}
(III)		10 ^{5.3}
pPLA-HFIF-67-12 Δ 19 (42 °C) (I)		10 ^{4.5}
Control HF-IF in extract of M5219/pPLA8 (42 °C)		10 ^{4.7}
pPLC-HFIF-67-8 (42 °C) (II): elution peak A		10 ^{4.5} ; 10 ^{4.2}
elution peak B		10 ^{4.5} ; 10 ^{4.7}
Control HF-IF eluted from polyacrylamide gel		10 ^{5.0}
Control HF-IF		10 ^{5.7}
Control leukocyte IF		<10
<i>g</i> , Anti-viral activity in heterologous cells (see Table 3)		
<i>h</i> , 2-5A synthetase induction (see Table 4)		

The following experimental methods were used for the characterization: *a*, dialysis at neutral pH took place overnight at 4 °C against phosphate buffered saline (PBS). *b*, Two volumes of a saturated (NH₄)₂SO₄ solution were added to one volume of extract. After 30 min on ice, the pellet was centrifuged at 12,000g for 10 min and redissolved in PBS. *c*, The bacterial extracts were either dialysed for 15 h against glycine-HCl (50 ml, pH 2.2), followed by dialysis against PBS for 3 h, or acidified with HCl, followed by neutralization with NaOH. After removal of the precipitate the antiviral activity was determined. *e*, Trypsin digestion was for 1 h at 37 °C with serial dilutions of the enzyme added to the diluted extract. The lowest trypsin concentration that completely abolished the antiviral activity is indicated. *f*, The antibody neutralization assays were carried out essentially as described by Havell *et al.*²². About 10 IF units ml⁻¹ of the preparations were incubated for 1 h at 37 °C with serial dilutions of goat anti-HF-IF antiserum, after which the residual antiviral activity was determined. Values are presented as neutralizing titres, that is, the highest dilution of antiserum which neutralized the protective effect of IF by 50% multiplied by the interferon titre of the sample assayed. Roman numerals in parentheses refer to the extraction methods described in Table 1.

Table 3 Antiviral protection of bacterial IF activity and authentic interferons

	Interferon activity (units per ml), assayed on					
	Human T-21	Human VGS	Monkey BSC-1	Rabbit primary kidney	Feline lung	Mouse L-929
M5219/pPLc-HFIF-67-8 (42 °C) (II)	3,000	300	<100	<100	<100	ND
(III)	30	<10	<10	<10	<10	ND
M5219/pPLc-HFIF-67-8 (42 °C) (II) elution peak A	2,000	200	<10	<10	<10	<10
M5219/pPLc-HFIF-67-8 (42 °C) (II) elution peak B	2,000	500	<10	<10	<10	<10
Human fibroblast interferon	3,000	500	30	30	<3	<2
Human leukocyte interferon	5,000	500	30	10	1,000	30
Human immune (type II) interferon	3,000	1,000	10	<3	<3	<2
Mouse L-929 interferon	<2	<2	<2	<2	<2	500

The antiviral activity was assayed as described in the legend to Table 1, except that the titres were directly determined from the dilution end points. T-21 are human fibroblasts trisomic for chromosome 21; VGS are normal human diploid fibroblasts²³. Feline lung cells were obtained from Flow Laboratories (cat. no 0-10907). ND, not determined.

which resulted in a different migration of the proteins, as revealed by internal ¹⁴C-labelled protein markers. Both peaks were neutralized to the same extent with anti-HF-IF antiserum (Table 2) and did not show detectable IF activity on heterologous cells (Table 3). The first peak, corresponding to an approximate molecular weight (MW) of 15,000–18,000, may have arisen by haphazard proteolytic cleavage of the fusion protein, or by limited *bona fide* processing at the now internal signal peptide, or by a combination of both processes. As shown previously, the absence of the carbohydrate moieties results in a protein which migrates in polyacrylamide gel to a position of about 4,000 MW below the authentic glycosylated HF-IF¹⁰. The 15,000–18,000-MW component, clearly present in *E. coli* M5219/pPLc-HFIF-67-8 extract, could also be detected at low but still significant levels in M5219/pPLa-HFIF-67-12Δ19 extracts. The second peak, with an apparent higher molecular weight, could correspond to the fused prokaryotic HF-IF poly-

peptide, or a slightly processed form. The tentative identification of the slower moving HF-IF activity peak as the fusion protein is strengthened by a different migration of the activity in the extract of M5219/pPLa-HFIF-67-12Δ19 (with a predicted fusion protein of about 28,000 MW) compared to the M5219/pPLc-HFIF-67-8 extract (with a predicted fusion protein of about 33,000 MW) (Fig. 2). The fusion proteins may themselves have some activity or be processed to an active product at the time when the bacterial extract (or gel eluate) is applied onto the human cells for the antiviral assay.

Conclusion

We have demonstrated the expression of HF-IF activity in *E. coli*. This synthesis depends on the presence of the HF-IF cDNA gene in the appropriate orientation and of the controlled induction of transcription from the P_L promoter. The antiviral activity obtained from *E. coli* is due to the presence of polypeptide(s) which for all physicochemical, biological and immunological characteristics tested closely resembles authentic HF-IF. Polyacrylamide gel electrophoresis resolved the bacterial IF activity into two different size classes; the smaller component presumably resulted from a post-translational cleavage of the fusion proteins.

The HF-IF produced in *E. coli* is still low in titre (about 100 units per 5 × 10⁸ cells ml⁻¹), but undoubtedly this can be improved by better plasmid constructions. Thus, we hope to produce sufficient quantities of bacterial HF-IF to compare its biological and pharmacological properties with those of authentic glycosylated HF-IF and of bacterial leukocyte IF, and perhaps to evaluate its potential clinical applications.

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Table 4 2-5A synthetase inducing activity of bacterial lysates and its neutralization by anti-HF-IF antiserum

	³² P incorporation (in c.p.m.) into the 2-5A trimer (background subtracted)
a, M5219/pPLc-HFIF-67-8 (42 °C) (III) (30 units ml ⁻¹)	3,618
Control HF-IF added to M5219/pPLa8 (42 °C) (III) (30 units ml ⁻¹)	3,695
M5219/pPLa8 (42 °C) (III) (<2 units ml ⁻¹)	(–1,360)
Control HF-IF (18 units ml ⁻¹)	1,120
(60 units ml ⁻¹)	4,338
(180 units ml ⁻¹)	10,273
(600 units ml ⁻¹)	21,698
b, Control HF-IF (100 units ml ⁻¹)	12,468
M5219/pPLa8 (42 °C) (I)	1,011
M5219/pPLa-HFIF-67-12Δ19 (42 °C) (I)	42,193
plus 1 n.u. per ml anti-HF-IF	29,260
plus 100 n.u. per ml anti-HF-IF	727
M5219/pPLc-HFIF-67-8 (42 °C) (I)	17,478
plus 1 n.u. per ml anti-HF-IF	12,115
plus 100 n.u. per ml anti-HF-IF	7,992
plus 10000 n.u. per ml anti-HF-IF	2,517

All samples were diluted sixfold (a) or tenfold (b) before assay. Antiviral titres (before dilutions) are given in parentheses. Neutralization with goat anti-HF-IF antiserum was at 37 °C for 1 h; n.u.s. neutralizing units. Roman numerals in parentheses refer to the extraction methods described in Table 1 legend. The obtained values are listed after subtraction of the endogenous background activity: 3,342 c.p.m. in a and 2,073 c.p.m. in b. The 2-5A synthetase assay was modified from Kimchi *et al.*²⁴ and Minks *et al.*²⁵. Confluent monolayers of HeLa cells in microtitre plates (96 wells) were treated with the diluted bacterial extract or with control HF-IF for 20 h. After cooling and washing with NaCl (140 mM), Tris-HCl (35 mM, pH 7.5), the cultures were lysed in 5 µl (a) or 10 µl (b) of 0.5% NP40, PMSF (1 mM), NaCl (140 mM), Tris-HCl (35 mM, pH 7.5). After shaking vigorously for 20 min at 0 °C, the lysates were collected and centrifuged for 20 min at 10,000g. 3.5 µl of the supernatant was incubated for 2 h at 31 °C in 6 µl of KOAc (100 mM), Mg(OAc)₂ (25 mM), HEPES-KOH (10 mM, pH 7.4), ATP (5 mM), fructose-1,6-bis-phosphate (4 mM), DTT (1 mM), poly I-C (20 µg ml⁻¹) and 2 µCi of lyophilized [α -³²P]ATP (400 Ci mmol⁻¹). The reaction was stopped by heating for 3 min at 95 °C and the samples were treated with 150 units ml⁻¹ of calf intestine alkaline phosphatase (Boehringer) at 37 °C for 1 h. After clearing, 1 µl was spotted on PEI-cellulose thin-layer plates and chromatographed in 1 M acetic acid for 2–3 h. The plates were autoradiographed and the incorporation of ³²P in the 2-5A trimer was determined.

Nucleotide sequence of an avian sarcoma virus oncogene (*src*) and proposed amino acid sequence for gene product

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The transforming gene (src) of avian sarcoma virus (ASV) and adjacent regions of the viral genome have been isolated by molecular cloning of viral DNA. Their nucleotide sequence encompasses the whole of src and the portion of the gene env that encodes gp37, one of two glycoproteins found in the viral envelope. Src encodes a single, hydrophobic protein with structural features that conform to previous descriptions of the gene product (pp60^{src}). It appears that a single viral protein is responsible for both the initiation and maintenance of neoplastic transformation by avian sarcoma virus. Neither src nor its product bear any obvious structural relationship to several other viral oncogenes and their encoded proteins. Src is flanked by a repeated nucleotide sequence that may facilitate frequent deletion of the gene from the viral genome.

VIRAL oncogenesis takes two general forms. Infection by some tumour viruses is apparently one of several successive events that combine to produce the neoplastic phenotype¹. The precise role of the virus in this setting is an enigma. Other viruses bear genetic loci ('transforming genes' or 'oncogenes') whose expression may be wholly responsible for the transformation of infected cells². Successful studies of both forms of viral oncogenesis have been carried out, but we owe much of the recent advance in our understanding of neoplastic transformation to the exploration of viral oncogenes—their action on the cell is swift and direct; they can be mutated to obtain useful conditional defects; the nucleic acids of which they are composed can be isolated and sequenced; and they act by means of proteins whose properties must underlie the mechanism of transformation.

Avian sarcoma virus (ASV) is a retrovirus that induces fibrosarcomas in infected hosts and transforms fibroblasts in culture. The genome of ASV carries a genetic locus (*src*) whose action is required for both the initiation and maintenance of neoplastic transformation³. Previous work has identified one product of *src*—a phosphoprotein of molecular weight 60,000 (refs 4–6) that is anchored to the cytoplasmic aspect of the plasma membrane of infected cells^{7,8}. Both crude⁹ and purified^{10,11} preparations of pp60^{src} phosphorylate tyrosine exclusively in protein substrates, and cells transformed by ASV contain more phosphotyrosine than do uninfected cells^{9,12}. These findings have engendered the hypothesis that phosphorylation of tyrosine in one or more cellular proteins is central to the mechanism by which *src* transforms cells. Important issues remain unresolved, however. Can the action of *src* alone induce neoplastic transformation? Is pp60^{src} the sole protein encoded by *src*? What is the nature of the association between pp60^{src} and the plasma membrane? Where are the phosphoamino acids located in pp60^{src}, and what is their role in the enzymatic activity of the protein? Does the phosphotransfer carried out by pp60^{src} *in vitro* reflect the activity of the protein in the infected cell? Might pp60^{src} possess functional properties other than protein kinase activity? As a step towards the resolution of these issues, we have determined the nucleotide sequence of *src* and regions adjacent to it in the ASV genome, and we have used these data to deduce a possible amino acid sequence for pp60^{src}. Inspection of the derived protein sequence permits some speculations on possible relationships between structure and function of pp60^{src}.

Experimental approach

The RNA genome of retroviruses is transcribed into duplex DNA during the early hours of infection¹³. Circular forms of retrovirus DNA can be isolated from infected cells and cloned into prokaryotic vectors^{14,15}. We have described previously the use of molecular cloning to isolate a 3.1-kilobase *Eco*RI fragment of DNA (see Fig. 1) that contains the entirety of *src*, all but 65 nucleotides of *c* (an untranslated region that lies to the right of *src* on the genetic map of ASV; see ref. 16), and about 1.0 kilobases to the left of *src*^{15,17}. We subcloned this DNA into the single-stranded DNA phage M13mp2 (ref. 16) to facilitate sequence analysis by the chain termination technique developed by Sanger and colleagues¹⁸. Clones representing both the positive and negative strands of viral DNA were isolated and sequenced so as to ensure the accuracy of the results. We also

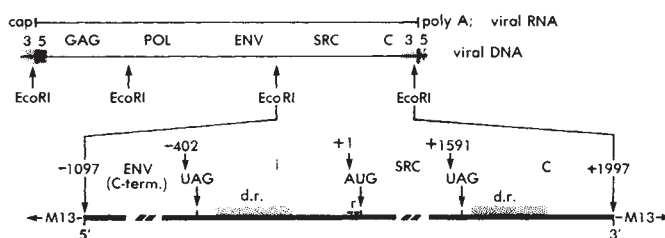
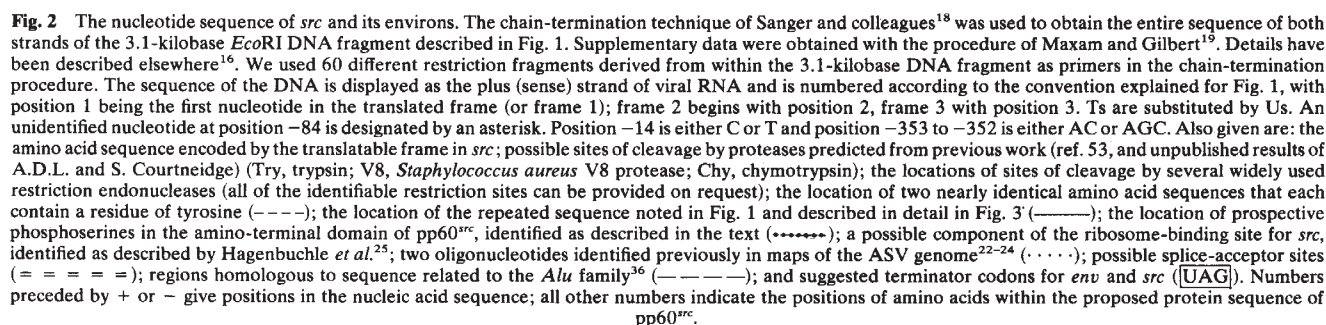


Fig. 1 The genome of ASV and the topography of the subclone used for nucleotide sequencing. A 3.1-kilobase *Eco*RI fragment of cloned ASV DNA was inserted into the genome of M13mp2 at the *Eco*RI site of the phage genome and used for sequencing, as described in detail elsewhere^{16,17}. The figure locates the boundaries of the subcloned fragment on the map of the ASV genome and outlines the topography of the fragment deduced from the nucleotide sequence illustrated in Fig. 2. The viral genome is given in both its RNA and DNA forms; the latter has a terminal redundancy composed of nucleotide sequences derived from both the 3' and 5' ends of the viral RNA^{20,22}. The identified genes of ASV are *gag* (structural proteins of the interior of the virion), *pol* (reverse transcriptase), *env* (glycoproteins of the virion envelope), and *src* (the oncogene). Positions on the nucleotide sequence were numbered with respect to the first nucleotide in *src* (+1). C-term denotes the putative carboxy-terminal domain of *env*. A repeated sequence described in the text is denoted as d.r.; this repeat is direct, that is, it occurs in the same orientation at each position (see Fig. 3). A domain that apparently separates *env* from *src* is denoted *i* for intergenic, and an untranslated region at the 3' end of the viral genome is denoted *c* (refs 16, 17). A possible component of the ribosome-binding site for *src* (see Fig. 3) is designated *r*.



used the procedure of Maxam and Gilbert¹⁹ to obtain supplementary data. The complete strategy for the sequencing and all data are available on request.

Authentication of the nucleotide sequence

Figure 2 illustrates the entire sequence of the 3,094 nucleotides that comprise the 3.1-kilobase pair *EcoRI* DNA fragment, displayed as the sense strand of the viral RNA. Positions within the sequence are numbered with respect to the first nucleotide in *src* (+1) (see below). The nucleotide composition of the fragment is 21.1% T, 25.7% dC, 23.4% dA, and 29.8% dG. We have a variety of reasons to believe that our nucleotide sequence for *src* and its environs is correct:

(1) We have shown elsewhere that the cloned *EcoRI* DNA fragment inserted into M13 for purposes of sequencing contained a functionally competent *src* gene²⁰; hence, the structure of *src* (and by inference, the remainder of the DNA fragment) was apparently undisturbed by cloning and propagation in prokaryotic vectors.

(2) The ASV DNA from which we prepared our molecular clones has been mapped extensively by site-specific cleavage with restriction endonucleases^{15,21}. The sequence illustrated in Fig. 2 contains all the sites located previously by this means. During the preparation of more than 60 primers for sequencing with the terminator technique, we identified a number of additional restriction sites; these also appear at appropriate positions in the final sequence. Figure 2 summarizes the locations of cleavage sites for several widely used restriction endonucleases. A complete catalogue of restriction sites within the 3.1-kilobase *EcoRI* fragment is available from the authors on request.

(3) The boundaries that we propose for *src* and *env* are in accord with previously developed physical maps of the ASV genome (see below).

(4) Previous analyses of the ASV genome have identified a number of T1 oligonucleotides that also appear in the nucleotide sequence reported here. Moreover, our data are in general agreement with previous, less precise efforts to locate these oligonucleotides on the ASV genome. For example, the oligonucleotide at positions -436 to -420 is contained within the carboxy-terminal domain of *env*, as suggested previously²², and the oligonucleotide at positions +1,601 to +1,612 lies just beyond the 3' boundary of *src*, rather than within the gene²³. Other correlations with our data include: six large oligonucleotides within *src* that were identified previously in the genome of SR-A ASV²³, and four oligonucleotides in the vicinity of the carboxy-terminus of *env*, three of which occur in the genome of the Prague-B strain of ASV, the fourth in the genome of the endogenous chicken virus, RAV-0 (ref. 24). The details of these correlations are omitted here but will be supplied on request.

(5) The proposed nucleotide sequence of *src* encodes a protein with structural features that conform to previous descriptions of pp60^{src} (see below).

Structural domains within the nucleotide sequence

Computer-assisted analysis of the sequence allowed us to identify several structural domains, which are discussed below and outlined in Fig. 1.

Positions +1 to +1,590 contain a single open reading frame, initiated by AUG and terminated by an amber codon (UAG, (positions +1,591 to +1,593)). We deduce that this sequence constitutes *src*: it is located in the appropriate position on the physical map of the ASV genome¹⁷, and translation from the open frame would generate a protein with structural features that conform to previous descriptions of pp60^{src} (see below). Translation from the other two reading frames in the *src* domain is blocked principally by opal codons (UGA; 11 of 13 terminators in frame 2, 12 of 15 terminators in frame 3), an unusual feature not encountered in other portions of the DNA fragment. In the unlikely event that all of the 11 opal terminators were to be suppressed, a protein with a molecular weight of ~44,000 could arise from positions +599 to +1,805; we have no reason to

believe that this ever happens in the infected cell. Of the 16 nucleotides between positions -21 and -6, 10 can base-pair with the nucleotide sequence at the 3' terminus of 18S ribosomal RNA (see Fig. 3). Hagenbuchle *et al.* have suggested that base-pairing of this sort may be a general feature of the sites at which translation initiates in eukaryotic cells²⁵. A similar structure can be formed near the 3' terminus of *src* (positions +1,341 to +1,357), but we cannot yet attribute any specific function to this location.

Positions -1,097 to -401 also contain a single open reading frame that begins at the third base at the left end of the DNA fragment (position -1,095) and terminates with an amber codon (position -402 to -400). The other reading frames are blocked by a mixture of termination codons. We presume that this domain represents the carboxy-terminal portion of the *env* gene that encodes the glycoproteins of the ASV envelope²⁶. The location of this domain on the viral genome is appropriate for *env*^{17,21}, and the amino acid sequence deduced from the open reading frame conforms to available information on the amino acid sequence of a protein (gp37) encoded by *env* (see below).

Positions -399 to -1 are closed to translation in all reading frames and apparently constitute an intercistronic region (i in Fig. 1) that separates *src* from *env*. Notable features of this region will be discussed below.

Positions +1,594 to +1,997 comprise an apparently untranslated domain previously designated as c^{16,17}. We have described the sequence of this domain in detail elsewhere¹⁶.

Miscellaneous aspects of the nucleotide sequence

Several nucleotide sequences are repeated in the untranslated regions on either side of *src*. The most striking of these is a sequence of 126 nucleotides (designated d.r. in Figs 1-3) that is repeated directly (that is, in the same orientation) with 79% fidelity at positions -317 to -200 and +1,685 to +1,808, and that can be extended to positions -377 and +1,632 with a fidelity of 63%.

A purine-rich sequence of 13 nucleotides occurs juxtaposed to the 3' end of d.r. Other work has implicated this sequence in the initiation of 'plus strand' DNA synthesis by reverse transcriptase^{27,28}. Similar purine-rich sequences are found 11 times at fairly regular intervals (~100-250 nucleotides) throughout the 3.1-kilobase fragment. Perhaps some of these sequences are also involved in the synthesis of plus strand viral DNA, which apparently initiates at multiple sites along the viral genome^{29,30}.

The 3.3-kilobase mRNA for *src*³¹ is formed by joining approximately 0.36 kilobases from the 5' end of the viral genome to a point located to the left (to the 5' side) of the *src* gene^{32,33}. From the size of the mRNA and the length of the spliced leader, we compute that the splice junction should lie about 0.5 kilobases to the 5' side of *src*—possibly within the carboxy-terminal domain of *env*³⁴. We therefore searched the appropriate regions of the 3.1-kilobase DNA fragment and found four sequences that resemble the proposed prototype (5'-PyPyNPYAG-3') for splice acceptor sites³⁵: TTGTAG at positions -509 to -504 (apparently within the carboxy-terminal domain of *env*), CCTAG at positions -232 to -227 and -177 to -172, and CTGAG at positions -62 to -57 (located within the i region). We have no means of implicating any of these sequences in splicing. Direct analysis of viral mRNA will be required for definitive identification of the splice junction.

Positions -140 to -128 and +54 to +67 resemble sequences found within the *Alu* family³⁶ of interspersed repeated sequences in mammalian DNA and at the origins of replication for several viral DNAs (Fig. 3). The function of these widely distributed nucleotide sequences is not known. One hypothesis suggests that they may serve as origins of DNA replication in the mammalian genome³⁶. If so, their presence in the nucleotide sequence of the ASV genome may be gratuitous: retrovirus DNA probably does not function as an independent replicon and may therefore have no need for a replication origin^{37,38}. Alternatively, the *Alu* sequences might participate in the

Table 1 Distribution of individual amino acids within the deduced sequence of pp60^{src}

Position	Phe	Leu	Ile	Val	Trp	Pro	Ala	Gly	Met	Cys	Tyr	Ser	Thr	Asn	Gly	His	Lys	Arg	Glu	Asp	Hph	b-s	OH
1-60	0	2	0	0	1	14	4	4	1	0	0	12	7	0	3	0	3	6	1	2	21	6	19
61-120	0	8	0	3	1	7	0	6	0	1	0	8	9	1	1	1	3	10	1	0	19	13	17
121-180	0	7	1	2	1	7	4	6	2	0	0	8	6	2	3	0	2	8	1	1	22	8	14
181-240	2	5	3	4	0	2	5	4	0	1	4	5	2	3	4	2	7	4	0	3	21	10	7
241-300	0	6	1	2	2	3	7	9	0	2	0	3	3	1	3	4	3	4	2	5	21	4	6
301-360	2	7	3	6	0	2	4	4	3	0	4	3	0	0	3	3	6	2	7	1	24	3	3
361-420	1	7	3	6	0	2	5	3	3	3	3	1	1	4	1	1	6	4	5	24	-1	2	
421-480	3	5	3	4	2	4	5	6	1	0	2	2	5	1	1	0	4	5	4	2	26	3	7
481-530	1	6	0	2	1	6	5	3	2	5	1	3	0	0	3	2	0	3	4	2	21	-1	3
Total no.	9	53	14	29	8	47	39	45	12	12	14	45	33	13	22	13	29	48	24	21	199	—	77
%	1.7	10	2.6	5.5	1.5	8.9	7.4	8.5	2.3	2.3	2.6	8.5	6.2	2.5	4.2	2.5	5.5	9.0	4.5	3.9	37.5	—	14.5

The amino acid sequence illustrated in Fig. 2 was divided arbitrarily into domains of 60 amino acids, beginning at the amino-terminus. The table gives, for each domain, the total residues of each amino acid, the sum of the hydrophobic residues (Phe, Leu, Ile, Val, Trp, Pro and Ala; Hph), the predominance of basic over acidic amino acids in each domain (computed as $b-s$; the difference between the sum of His, Lys and Arg and the sum of Glu and Asp), and the sum of the hydrophilic amino acids Ser and Thr (OH).

maturation of mRNAs³⁶. We note that three of the candidate splice junctions for *src* mRNA are located in the vicinity of the *Alu*-like sequence at positions -140--128.

The amino acid sequence of pp60^{src}

We used the nucleotide sequence of the open reading frame in *src* to deduce the sequence of the 530 amino acids contained in the encoded protein. Figure 2 illustrates the deduced amino acid sequence, and Table 1 describes the distribution of individual amino acids within the protein. Several features of the deduced structure conform to previous descriptions of pp60^{src}: the molecular weight is 58,449; the protein is hydrophobic¹¹; 9 of the 12 methionines are in the carboxy half of the molecule (ref. 39, and H. Oppermann, personal communication); and the amino acid sequence can account for seven sites of specific cleavage by proteases predicted from previous work (Fig. 2). We conclude that we have probably determined the authentic amino acid sequence for pp60^{src}.

Two separate functional domains have been defined within pp60^{src} (refs 11, 40). One domain (confined to a molecular weight of ~15,000 at the amino-terminus of the protein) anchors the protein to the cytoplasmic aspect of plasma membrane, the other domain protrudes from the membrane and

carries the protein kinase activity. These functional domains have apparent correlates in the composition of pp60^{src} (see Table 1): Pro, Arg, Ser and Thr are concentrated in the amino-terminal domain, Cys, Met and Tyr in the carboxy-terminal domain. Most of the protein (and the amino-terminal half in particular) is basic, whereas the carboxy-terminal domain is relatively acidic. We presume that the one-dimensional topography of pp60^{src} described here has its counterparts in the three-dimensional configuration of the protein (which we already know to be a highly elongated molecule—frictional coefficient 1.4; see ref. 5). It seems that the protein was designed on the one hand for tethering to the plasma membrane, and on the other for enzymatic activity beyond the confines of the membrane.

Using conventional procedures⁴¹, we have deduced general features for the possible secondary structure of pp60^{src}. The central portion of the molecule (amino acid residues 221-438) is likely to be in the α -helical configuration; the carboxy-terminal domain seems to be composed of alternating α -helices and β -pleated sheets; and the amino-terminus is probably mainly β -sheet.

The portion of pp60^{src} that is anchored to the plasma membrane contains one (or more) residues of phosphoserine^{6,9},

Direct repeat (d.r.):

- 377 to - 189 (i): 5'-GCTGAAGAGCGGATAGTATAGGACAGTACATGGT-3'
+1632 to +1819 (c): 5'-ACGACAAATTC AT GAAGAA TCTGCTTA GGTTAGGCG TTTTGCGCTGCT TOG -3'

continued: i: 5'-CGTATATACGCCAT-3'
c: 5'-CG ATGTACGGGCGATATACCGGTATCTGAGGGGACTAGGCTGTGTTTAGGCGAAAGGG -3'

continued: i: 5'-GGTCAAGGTTGTAGCGGTTAGGAGTCCCTTAGGATATAGTACAGCGCTTTTCATATGAT-3'
c: 5'-GGCTTGGTTGTAGCGGTTAGGAGTCCCTTAGGATATAGTACTTTTGGCTTTTCATAGGGA -3'

continued: i: 5'-ACATAACTT-3'; 188 nucleotides to AUG of *src*
c: 5'-GGGGAAAT-3'; 246 nucleotides to polyA addition site

18S ribosomal RNA binding site:

- 21 to - 6 5'-TGGGAGTGGAGGG A-3'
3'-AUUAC UAGGAAGGCGU -18S rib. RNA-5'

+1341 to +1357 5'-TGTCTGGTCTTGGCA -3'
3'-AU UACUAGGAAGGCGU -18S rib. RNA-5'

Acceptor-site consensus sequence: 5'-PyPVPYAG-3'

-509 to -504 5'-TTGTAG-3' in env-region
-232 to -227 5'-CCITAG-3' in "i"-region
-177 to -172 5'-CCITAG-3' in "i"-region
-62 to -57 5'-CTGCAG-3' in "i"-region

"Alu-family" and related sequences:

Alu Family: 5'-GGAGGCTGAGGCAG-3'
Pol III γ -Globin Template: 5'-GGAAGCTGAGGAG-3'
CHO Low Molecular Weight RNA: 5'-GGAGGCAGAGGCAG-3'
BK Virus Origin: 5'-GGAGGCAGAGGCGG-3'
SV 40 Origin: 5'-AGAGGCGAGGCGG-3'
Polyoma Virus Origin: 5'-AGAGGCGAGGCGG-3'
Hepatitis B Virus: 5'-GGAGGCAGAGGCAG-3'
ASV RNA: 5'-GGAGGCAG CTGG-3'
Position -140 to -128: 5'-GGAGGCAG CTGG-3'
Position +54 to +67: 5'-GGAGGCAGAGGCAG-3'

Fig. 3 Repeated nucleotide sequences within and without *src*. The figure illustrates the various repeated elements identified in the nucleotide sequence of the 3.1-kilobase *EcoRI* DNA fragment. The illustrated sequences include: the direct repeat that occurs on both sides of *src*; the consensus sequence from the *Alu* family of interspersed repeats in mammalian DNA and variants of this sequence noted by Jelinek *et al.*³⁶; a possible component of the ribosome-binding site for *src* and a similar sequence found near the 3' terminus of the gene; and possible splice-acceptor sites located to the 5' side of *src*.

Table 2 Frequency of codons used in the *src* gene

3/UUU/Phe	5/UCU/Ser	2/UAU/Tyr	1/UGU/Cys
▶ 6/UUC/Phe	8/UCC/Ser	▶ 13/UAC/Tyr	▶ 11/UGC/Cys
1/UUA/Leu	7/UCA/Ser	0/UAA/OC	0/UGA/OP
5/UUG/Leu	8/UCG/Ser	▶ 1/UAG/AM	8/UGG/Trp
8/CUU/Leu	9/CCU/Pro	5/CAU/His	1/CGU/Arg
▶ 13/CUC/Leu	▶ 15/CCC/Pro	8/CAC/His	▶ 12/CGC/Arg
1/CUA/Leu	10/CCA/Pro	6/CAA/Gln	2/CGA/Arg
▶ 26/CUG/Leu	▶ 12/CCG/Pro	▶ 16/CAG/Gln	▶ 19/CGG/Arg
3/AUU/Ile	7/ACU/Thr	1/AAU/Asn	5/AGU/Ser
▶ 11/AUC/Ile	▶ 11/ACC/Thr	9/AAC/Asn	▶ 12/AGC/Ser
0/AUA/Ile	▶ 11/ACA/Thr	4/AAA/Lys	9/AGA/Arg
12/AUG/Met	4/ACG/Thr	▶ 25/AAG/Lys	5/AGG/Arg
4/GUU/Val	▶ 11/GCU/Ala	8/GAU/Asp	5/GGU/Gly
7/GUC/Val	▶ 16/GCC/Ala	▶ 14/GAC/Asp	▶ 15/GGC/Gly
2/GUA/Val	7/GCA/Ala	6/GAA/Glu	9/GGA/Gly
▶ 16/GUG/Val	5/GCG/Ala	▶ 19/GAG/Glu	▶ 16/GGG/Gly

Relatively abundant codons are indicated by the arrowheads.

the exposed (kinase) domain contains phosphotyrosine⁹. We have attempted to identify the possible locations of these phosphoamino acid sequences in the deduced amino acid of pp60^{src}. Phosphoserines in other proteins are usually found in the following general structure: basic amino acid–basic amino acid–any amino acid–phosphoserine⁴². Three serine residues (17, 73 and 83) in the amino-terminal domain of pp60^{src} satisfy this criterion. Two tyrosine residues in the carboxy half of the sequence (379 and 385) are situated within virtually identical microenvironments (Arg-Met-X-Tyr-Val, where X is either Ala or Asn) that are separated by a single amino acid (Glu). We speculate that this unlikely repetition of amino acid sequence might denote the position(s) of phosphotyrosine in pp60^{src}.

Codon usage in *src*

Table 2 illustrates the prevalence of various codons for each of the amino acids in the translated frame of *src*. Relatively strong biases for particular codons were observed in several instances: CUC and CUG for Leu; UGC for Cys; AUC for Ile; GUG for Val; CAG for Gln; GAG for Glu; UAC for Tyr; CGG and CGC for Arg; and GGC and GGG for Gly; AAG for Lys. U and A are used relatively infrequently in the third position with the exception of the codons for Thr and Pro.

A possible amino acid sequence for the carboxy-terminal domain of viral glycoprotein

The *env* gene of ASV encodes two glycoproteins (gp85 and gp37) which originate from the cleavage of a single polypeptide precursor and remain linked to one another by disulphide bonds²⁶. The proteins are arranged 5'-gp85-gp37-3' on the gene. It seems possible that we have obtained the sequence of 697 nucleotides at the 3' end of *env*, or 231 amino acids extending leftward from the carboxy-terminus of gp37 (Fig. 4). Hunter and his colleagues have identified eight amino acid residues in the amino-terminal region of gp37 of the Prague-B ASV, a different strain of ASV (E. Hunter, personal communication). Four of the identified residues occur at appropriate positions in the sequence proposed by Fig. 4: Thr at position 43, Asp at positions 47 and 48, and Glu at position 53 (residues 2, 6, 7 and 12, respectively, in the amino acid sequence, from E. Hunter *et al.*, personal communication). The codons for two other residues could be generated by changes in single nucleotides, converting Cys to Gly at position 50 in Fig. 4, and Ser to Ala at position 51. These correlations suggest that the amino acid sequence illustrated by Fig. 4 includes the entirety of gp37, whose amino-terminus would be located at residue 42 in Fig. 4, and whose un-glycosylated molecular weight would be 20,500. The carboxy-terminus of the deduced protein is exceptionally hydrophobic (37 of 43 amino acids, as noted in Fig. 4). This feature might denote a portion of gp37 that is embedded in the lipoprotein envelope of the virus and anchors the entire glycoprotein to the virion⁴³. The prevalences of individual codons

used to encode this protein are very different from those found for *src* (Table 3). Data presented elsewhere suggest that *src* and *env* had independent and very different evolutionary origins^{44,45}. The disparities in codon usage between the two genes may reflect their distinct origins.

Comparison of *src* with other viral oncogenes

Genetic loci responsible for neoplastic transformation of infected cells have been identified within the genomes of Moloney murine sarcoma virus, polyoma virus, SV40 virus and several strains of adenovirus (see ref. 2 for reviews). We have compared the available nucleotide sequences of these loci with the sequence of *src*, and the amino acid sequences of the encoded proteins with pp60^{src}. The nucleotide sequence of *src* bears little resemblance to the sequences of the other viral oncogenes, with the exception of a few oligonucleotides (the largest being nonamers) shared with the gene for the middle T antigen of polyoma virus. We found no appreciable homology among the various amino acid sequences, although pp60^{src}, the T antigen of polyoma, and two proteins encoded in the *E1b* region of adenovirus DNA are all relatively rich in proline (data not shown).

Further comments

Src is frequently deleted during the propagation of most strains of ASV³. The mechanism that facilitates this deletion has not been elucidated. We propose that the large repeated nucleotide sequence (d.r.) that flanks *src* might mediate deletion of the gene, for example, by homologous recombination within the viral genome⁴⁶. This proposal can be tested by analysing the sequence that remains at the site of the deletion subsequent to the loss of *src* (work in progress).

The direct determination of amino acid sequence remains a relatively arduous and demanding procedure, despite recent technical advances in sensitivity and convenience⁴⁷. It seems likely that the nucleotide sequence presented here provides the authentic amino acid sequence of pp60^{src} in advance of any direct sequencing of the protein. At least one issue remains controversial, however—the claim that a portion of pp60^{src} (presumably, a terminal 'signal sequence') is removed by membrane-bound protease(s) subsequent to synthesis of the protein⁴⁸. The issue can probably be resolved by identification of a few of the amino acids at the amino-terminus of the mature protein and comparison of these results with the nucleotide sequence of *src*. We note that amino-terminal signal sequences generally have hydrophobic domains rich in leucine⁴⁹, features not found in the amino-terminal region of the sequence of *src* (see Fig. 2).

The amino acid sequence deduced here for pp60^{src} suggests that the protein is hydrophobic (average hydrophobicity of 1.1, computed as described in ref. 50). This property is in accord with previous findings that pp60^{src} is an integral protein of the plasma membrane, can be released from the membrane only by treatment with detergents, and is more soluble in aqueous solutions when detergent is present¹¹. A more detailed analysis of the

→ gp 37 ?

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1 - IPSRFVGGPCYLGLKILMLAPKHTDILKVLVNSSRTGIRRKSTSHLDD - 48
49 - TCSDEVQLWGPTARIFASILSPGSSCEALRETERLACKSVKQANLIT - 96
97 - SLIGDILLDDVTSIRHVLQNRRAIDFLLAHGHGCEVAGMCCPNLSD - 144
145 - QSESIQKPKQLMKEHVNKIGVDSDLIGSNLRLFGGIGEMAVHLLKGL - 192
193 - UGLVWILLWLWCLFCLLQMLCGNRRRMINNSIELPHGI - 231

```

Fig. 4 A possible amino acid sequence for the carboxy-terminal domain of the glycoprotein of ASV. An amino acid sequence was deduced from the nucleotide sequence illustrated in Fig. 2, beginning with position -1,095 and ending before the amber codon at positions -402--400. The amino acid sequence is given in terms of the conventional single-letter symbols. The underlining demarcates an exceptionally hydrophobic region. The arrow denotes the presumptive amino-terminus of gp37.

Table 3 Codons used in C-terminal domain of the *env* gene

1/UUU/Phe	2/UCU/Ser	1/UAU/Tyr	3/UGU/Cys
4/UUC/Phe	2/UCC/Ser	0/UAC/Tyr	▶7/UGC/Cys
5/UUA/Ileu	4/UCA/Ser	0/UAA/OC	0/UGA/OP
▶9/UUG/Leu	1/UCG/Ser	▶1/UAG/AM	4/UGG/Trp
6/CUU/Leu	▶3/CCU/Pro	4/CAU/His	2/CGU/Arg
4/CUC/Leu	▶3/CCC/Pro	4/CAC/His	0/CGC/Arg
▶6/CUA/Leu	1/CCA/Pro	1/CAA/Gln	▶4/CGA/Arg
6/CUG/Leu	1/CCG/Pro	▶6/CAG/Gln	1/CGG/Arg
▶8/AUU/Ile	0/ACU/Thr	5/AAU/Asn	5/AGU/Ser
4/AUC/Ile	2/ACC/Thr	3/AAC/Asn	5/AGC/Ser
4/AUA/Ile	▶6/ACA/Thr	3/AAA/Lys	▶5/AGA/Arg
5/AUG/Met	1/ACG/Thr	▶8/AAG/Lys	1/AGG/Arg
4/GUU/Val	3/GCU/Ala	▶8/GAU/Asp	4/GGU/Gly
4/GUC/Val	4/GCC/Ala	4/GAC/Asp	5/GGC/Gly
3/GUA/Val	3/GCA/Ala	5/GAA/Glu	▶7/GGA/Gly
4/GUG/Val	2/GCG/Ala	4/GAG/Glu	5/GGG/Gly

Relatively abundant codons are indicated by arrowheads.

amino acid sequence has revealed two relatively isolated hydrophobic domains at residues 52–57 (average hydrophobicity 2.2) and 121–126 (average hydrophobicity 2.4) (data not illustrated). Both domains are located near the amino-terminus of the molecule where the protein apparently inserts into the plasma membrane^{8,40}.

The nucleotide sequence reported here substantiates previous assumptions that *src* encodes a single protein. Transfection of the 3.1-kilobase DNA fragment containing *src* into mammalian cells leads to the synthesis of pp60^{src} and the neoplastic transformation of recipient cells^{19,51}. Moreover, the transformed cells

are tumorigenic in newborn allogeneic animals (unpublished results of H. Oppermann and P. Luciw). We can now rigorously conclude that *src* is the only viral gene required for tumorigenesis by ASV, and that the mechanism of neoplastic transformation by the virus derives entirely from the biochemical properties of pp60^{src}.

Chemical analysis of pp60^{src} and site-specific mutagenesis of *src* will be used to identify the sites of functionally important modifications (such as phosphorylation) within the protein, to locate the active site for phosphotransfer by the protein, and to describe other important features of the protein. These efforts will require the nucleotide sequence of *src* and the amino acid sequence of pp60^{src}.

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Note added in proof: After preparing this manuscript, we learned of the identification of several new avian sarcoma viruses with transforming genes distinct from *src*. It is now more accurate to designate the virus studied here as Rous sarcoma virus, Schmidt–Ruppin strain—one of several isolates that share closely related *src* genes.

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DNA N-glycosylases and UV repair

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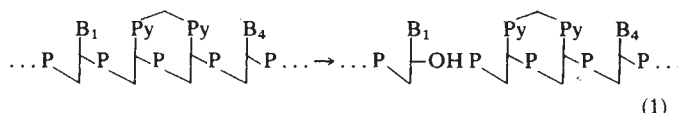
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Repair of some DNA photoproducts can be mediated by glycosylic bond hydrolysis. Thus, Escherichia coli endonuclease III releases 5,6-hydrated thymines as free bases, while T4 UV endonuclease releases one of two glycosylic bonds holding pyrimidine dimers in DNA. In contrast, uninfected E. coli apparently does not excise pyrimidine dimers via a DNA glycosylase.

DAMAGED bases can arise in DNA by a number of routes. These include spontaneous hydrolysis as in the deamination of cytosine¹ or adenine², reaction with such chemical agents as monofunctional alkylating agents³, and radiation.⁴ Two major

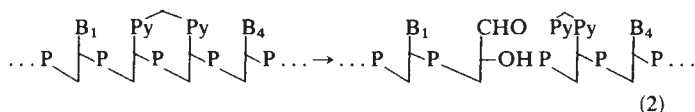
routes for repair of damaged DNA have been proposed for *Escherichia coli*, initiated by strand breakage near the damage⁵, or by specific hydrolysis of the glycosylic bond of a non-conventional base⁶. Using repair of pyrimidine dimers produced in

DNA by UV irradiation as a model system, a sequence of repair initiated by phosphodiester bond hydrolysis has been proposed⁷. In this scheme, the initial, perhaps rate-limiting step is endonuclease incision, mediated by enzymes specific for pyrimidine dimers as follows:



Excision of the damage is then carried out by an exonuclease, with DNA polymerase and DNA ligase restoring the integrity of the genetic material. Considerable attention has been directed recently toward the alternative pathway initiated by glycosylic bond hydrolysis. Four enzymes have been reported in *E. coli* which remove, respectively, uracil⁶, hypoxanthine², 3-alkyladenine⁸ or formamidopyrimidine⁹. This base excision results in the formation of apurinic or apyrimidinic (AP) sites, which could then be repaired by action of AP endonuclease, DNA polymerase and polynucleotide ligase.

Recently, Grossman and his colleagues presented evidence suggesting that UV endonuclease from *Micrococcus luteus* actually cleaves the phosphodiester bond between the nucleotides of a pyrimidine dimer¹⁰. This assertion was based on results of gel electrophoresis of a short fragment of UV-irradiated DNA incised by UV endonuclease. Products were observed which appeared to correspond to incision within the pyrimidine dimers. However, when these same fragments were heated at 100°C or exposed to alkali, they exhibited mobility corresponding to a strand cut immediately 5' to the pyrimidine dimer. This alkali- and heat-lability was similar to that observed for AP sites, and these authors interpreted their results to indicate that UV endonuclease cleavage occurs as follows:



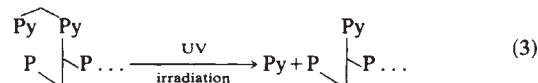
Thus, an AP deoxyribose is left at the 3' terminus of the nick and could be removed by heat or alkali to yield a DNA fragment shorter by apparently one nucleotide. This interpretation contrasted with previous models in which phosphodiester bond hydrolysis is the first step in the repair pathway and would result in cleavage 5' to the dimer rather than within it. We wished to examine which mechanism might apply to the UV endonuclease induced by infection of *E. coli* with bacteriophage T4. Like the *Micrococcus luteus* UV endonuclease, the T4 enzyme appears to be specific for pyrimidine dimers¹¹ and might well act by a similar mechanism.

In addition, we tested whether *E. coli* endonuclease III acts as a glycosylase followed by an AP endonuclease, since we had previously observed it to cleave two apparently unrelated substrates: DNA containing 5,6-dihydroxydihydrothymine and DNA containing AP sites¹².

T4 UV endonuclease has pyrimidine dimer glycosylase activity

We recently have taken advantage of the requirement of *E. coli* DNA polymerase I for a 3'-hydroxyl nucleotide terminus to probe the structure of endonuclease products¹³. In those experiments it was shown that the UV endonuclease controlled by phage T4 (gene ν) acts on UV-irradiated DNA to form nicks containing an AP deoxyribose at the 3' terminus. Such a structure could arise by hydrolysis of the glycosylic bond of the 5' pyrimidine nucleotide of the dimer, as proposed by Grossman *et al.*¹⁰ for the UV endonuclease from *M. luteus* (see above). The resulting pyrimidine dimer nucleotide at the 5' terminus would have an important feature: if the cyclobutane bonds of the dimer could be broken by additional UV irradiation (as demonstrated for free thymine dimers in aqueous solution¹⁴), one pyrimidine would be released as the free base from each such structure.

Concomitantly, a normal pyrimidine nucleotide would be left at the 5' terminus:



Thus, photolytic release of pyrimidine bases after treatment of UV-irradiated DNA with UV endonuclease could provide a rapid and specific assay for this unusual structure and for the action of a putative pyrimidine dimer-DNA glycosylase.

As seen in Fig. 1, incubation of UV-irradiated, ³H-thymidine-labelled DNA with T4 UV endonuclease did not of itself result in the release of free thymine. However, secondary irradiation of such enzyme-treated DNA did release free thymine base. The amount of base which could be released was a function of the primary dose to which the DNA was exposed; prolonged incubation with excess UV endonuclease did not increase the amount of free thymine formed. High pressure liquid chromatographic analysis of the products of a limit reaction did not

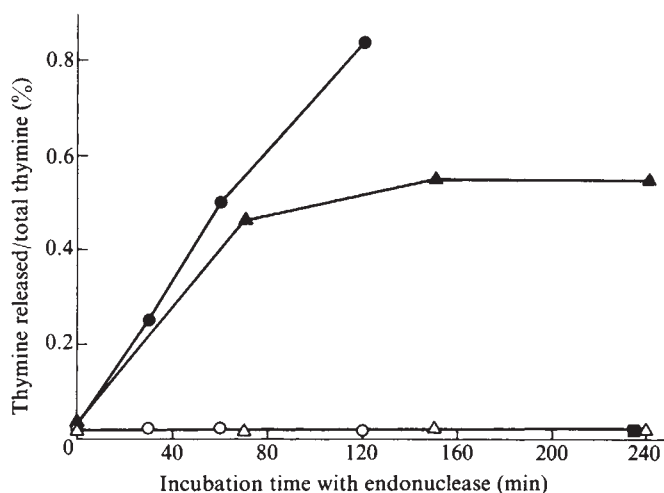


Fig. 1 Generation of photo-releasable thymine by T4 UV endonuclease. T7 ³H-DNA (25,000–29,000 c.p.m. per nmol nucleotide), prepared as described by Richardson³⁰, was irradiated on ice under a Westinghouse germicidal lamp (G15T8) at a dose rate of 3 J m⁻² s⁻¹, as determined by potassium ferrioxalate actinometry³¹. Irradiation was for 0 min (square), 10 min (triangles, 1.35 mM DNA-nucleotide), or 20 min (circles, 0.58 mM DNA-nucleotide). DNAs were then incubated at 37 °C for times indicated in 140 µl reactions containing 25 mM potassium phosphate, pH 7.4–1 mM EDTA–0.2 M NaCl–80 µg ml⁻¹ BSA, 17 nmol DNA-nucleotide and enzyme [8.4 µl per sample (squares, triangles) or 5.3 µl per sample (circles)]. After chilling, a second 20-min irradiation was carried out under conditions of the primary irradiations (>90% of maximum photorelease) (solid symbols). Samples were then assayed for thymine base by high pressure liquid chromatography (triangles) or by Dowex 1 chromatography (circles). For high pressure liquid chromatographic analysis samples were diluted to 300 µl with water and precipitated by addition of 30 µl of 1 mg ml⁻¹ *Bacillus subtilis* DNA and 900 µl 100% ethanol. After 10–30 min at –70 °C samples were spun for 5 min in a Beckman Microfuge. The supernatants were concentrated by evaporation, then mixed with 15 µl of 7.5 mM thymine and water added to a final volume of 350 µl. A 100-µl portion of each sample was then injected into a Waters model ALC/GPC 244 liquid chromatograph with C-18 reverse phase column (25 cm by 4.6 mm internal diameter) equilibrated with 2.5 mM potassium phosphate–2.5% methanol, pH 3.0³². Flow rate was 2 ml min⁻¹ at 1,500 p.s.i. The first 3 ml was collected as a single fraction; subsequent fractions were 0.3 ml. After determining the absorbance of the fractions at 254 nm to identify the thymine location, Triton X-100/toluene-based fluor was added to each and the ³H radioactivity determined. For thymine determination by Dowex 1 chromatography³³, samples diluted to 300 µl with water were precipitated by addition of 100 µl 10 mg ml⁻¹ BSA and 500 µl 7% TCA, and centrifugation. The supernatants were extracted twice with 13 vol. cold diethyl ether to remove TCA, and residual ether was removed under a stream of N₂, after which each sample was made to 1,100 µl with water and 11 µl 1 M Tris-HCl, pH 7.5. One ml of each sample was then applied to a 1 ml column of Dowex 1-X8 equilibrated with 10 mM ammonium formate, pH 4.2. The columns were washed with 5 ml of this same buffer in 1 ml aliquots, and 1 ml fractions were collected directly into scintillation vials and the radioactivity determined. T4 UV endonuclease was prepared as described by Friedberg and King³⁴, except that the DNA-cellulose fraction was stored at –20 °C in 50% glycerol containing 5 mM potassium phosphate, pH 7.0–0.5 mM dithiothreitol–5% ethylene glycol. Enzyme stored in this manner retained full activity after 2 yr.

Table 1 Generation of photo-releasable thymine and nicks by T4 UV endonuclease

Endonuclease incubation (min at 37 °C)	Secondary UV irradiation	Free thymine (pmol)	Total nicks (pmol)
0	—	<1	0.4
	+	<1	0.4
30	—	<1	2.0
	+	2.9	—
60	—	<1	4.2
	+	5.3	3.5
120	—	<1	*
	+	9.6	*

T7 ³H-labelled DNA (0.57 mM, 40,600 c.p.m. per nmol nucleotide) was irradiated with 1,800 J m⁻² UV light. Photo-released thymine formed by T4 UV endonuclease was measured by Dowex 1 chromatography as described in Fig. 1 legend. Nicking of the T7 DNA was determined by sedimentation in 5% to 20% sucrose gradients containing 0.25 M NaOH–5 mM EDTA for 210 min at 49,000 r.p.m. in a Beckman SW 50.1 rotor. Average molecular weights were calculated according to Studier³⁸ and used to determine the average number of nicks per T7 genome. Total nicks of 2 pmol correspond to 9.5 nicks per T7 genome.

* Too numerous to quantify.

detect any labelled base, nucleoside or nucleotide other than thymine with or without the second irradiation (data not shown).

The UV-irradiated DNA was also analysed for the number of nicks produced by UV endonuclease (Table 1). The number of nicks introduced during the enzyme incubation was approximately equal to or somewhat less than the amount of thymine releasable by the secondary irradiation. This near-equality might imply simultaneous action of the DNA glycosylase activity and phosphodiester bond cleavage. However, we have shown previously¹³ that, in an enzyme-limited reaction, T4 UV endonuclease acting on UV-irradiated DNA transiently creates sites which are sensitive to AP endonuclease. For T4 UV endonuclease therefore, pyrimidine dimer-DNA glycosylic bond cleavage appears to precede phosphodiester bond cleavage.

Pyrimidine dimers excised *in vivo* by uninfected irradiated *E. coli* are not in the form of pyrimidine dimer nucleotides

We also tested whether the excision of pyrimidine dimers in uninfected *E. coli* cells might be mediated by an *N*-glycosylase activity. Since the literature is unclear with respect to the properties of the enzyme(s) controlled by the *uvrA*, *uvrB* and *uvrC* loci^{15,16}, we chose to examine the structure of the products excised by this system *in vivo* from the DNA of irradiated cells. Setlow and Carrier¹⁷ and Boyce and Howard-Flanders¹⁸ have shown that pyrimidine dimers removed from the DNA of irradiated *E. coli* are contained in acid-soluble oligonucleotides which are retained in the cells. If this excision process is indeed mediated by hydrolysis of the glycosylic bond of one pyrimidine of the dimer, these oligonucleotides would be expected to contain the pyrimidine dimer nucleotide structure, which could be identified by its UV-dependent release of pyrimidine bases.

We therefore extracted acid-soluble oligonucleotides from cells that had been irradiated and then incubated for 3 h in growth conditions, during which 70% of the dimers were excised from the cellular DNA. Secondary irradiation of these oligonucleotides, however, did not release significant amounts of free thymine (Table 2). If the dimers in this material had each retained but a single glycosylic bond, half of the label measured in dimers should have been released as free thymine—2.2% of the total thymine for the sample shown. Instead, a UV dose which released thymine from essentially all of the dimers acted upon *in vitro* by T4 UV endonuclease yielded less than one-tenth of the 2.2% value. As a control for pyrimidine dimers retaining both glycosylic bonds, oligonucleotides were generated by pancreatic DNase digestion of DNA irradiated *in vitro*. This material (Table 2, sample 3) did not contain significant photoreleasable thymine. On the other hand, irradiated DNA which had been treated with excess T4 UV endonuclease prior to the pancreatic DNase digestion gave rise

Table 2 Test for photo-releasable thymine in excision products of UV-irradiated *E. coli*

Sample	Secondary UV irradiation (J m ⁻²)	Thymine in dimers (% total thymine)	Free thymine base (% total thymine)
(1) Acid-soluble fraction from irradiated, non-incubated cells	0	<0.01	0.33
	3,600	ND	0.35
(2) Acid-soluble fraction from irradiated, incubated cells	0	4.4	0.51
	3,600	2.5	0.68
(3) DNase I digest of irradiated T7 DNA, acid-soluble fraction	0	1.8	0.02
	3,600	5.9	0.05
(4) DNase I digest of irradiated, T4 UV endonuclease-treated T7 DNA, acid-soluble fraction	0	2.2	0.09
	3,600	6.2	1.11

E. coli AB1157 was grown for 3 generations to 10⁸ cells per ml in 100 ml K medium with 200 µg ml⁻¹ deoxyadenosine and 5 µCi ml⁻¹ ³H-thymidine (Amersham, 15 Ci mmol⁻¹). K medium contains per litre: 1 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 10 g glucose, 10 g vitamin-free casaminoacids, 0.2 mg thiamine, 24 mg MgSO₄ and 2 mg CaCl₂. Cells were collected and washed 5 times by centrifugation using 25 ml 0.1 M NaCl–10 mM Tris, pH 7.5 (NT buffer). The final pellet was suspended in 225 ml NT buffer and aliquots of 28 ml were each irradiated at 2 J m⁻² s⁻¹ for 30 s in a 15 cm glass Petri dish with mixing, then returned immediately to ice. Cells were collected by centrifugation and 1/4 of the irradiated cells was suspended in 1 ml 10 mM Tris, pH 7.5, and the remainder was suspended in 25 ml NT buffer and added to a flask containing 50 ml K medium. The flask was covered with foil to prevent photoreactivation and incubated for 3 h at 37 °C with shaking. After incubation, the cells were collected by centrifugation, washed twice with 25 ml NT buffer, then suspended in 1 ml 10 mM Tris, pH 7.5. An equal volume of cold 10% trichloroacetic acid (TCA) was then added to each cell suspension and, after mixing, incubated 10 to 15 min on ice. Acid-insoluble material was removed by centrifugation for 10 min at 10,000 g. The supernatants were extracted 3 times with 5 volumes of ice-cold diethyl ether. Remaining ether was removed with a stream of N₂. Free thymine in the acid-soluble extract of the incubated cells (1–2% of total ³H) was reduced by chromatography on DEAE-cellulose (Brown & Co., Berlin, New Hampshire). A sample (1.2 ml, 2 × 10⁵ c.p.m.) was diluted 5-fold with 10 mM Tris, pH 7.9 and applied at about 3 ml per h to a 3-ml column of DEAE-cellulose equilibrated with this same buffer. The column was washed with 5 ml of buffer and eluted with 10 ml of 0.5 M NaCl–9 mM Tris, pH 7.9 while 0.5 ml fractions were collected at 2 ml per h. Fractions containing about 98% of the applied radioactivity (3.0 ml) were pooled and evaporated to dryness at 35 °C. This material was redissolved in 300 µl distilled water and applied to a 0.7-cm × 38-cm column of Sephadex G10 equilibrated with 5 mM Tris, pH 8.2. Washing with this buffer was continued at 3 ml per h and 0.5-ml fractions collected. Radioactivity in the fractions was determined by scintillation counting, while NaCl was detected by conductivity measurement. More than 90% of the applied material was recovered free of NaCl and used for subsequent studies. Fifty-five µl T7 DNA (0.57 mM-nucleotide, 40,000 c.p.m. per nmol-nucleotide) was irradiated for 300 s at 3 J m⁻² s⁻¹ 254 nm light and 25 µl aliquots made up with stock reagents to 240 µl T4 UV endonuclease cocktail (see legend to Fig. 1). Thirty µl T4 UV endonuclease (33 fmol glycosylic bonds hydrolysed per min per µl) or 30 µl T4 UV endonuclease storage buffer were added and incubation carried out for 6 h at 37 °C. Endonuclease was inactivated by heating for 3 min at 70 °C and glycerol removed from the samples by dialysis against 1 litre of 10 mM Tris, pH 7.9. One-twentieth volume of 1 M Tris-HCl, pH 7.9 and 0.01 volume of 1 M MgCl₂ were then added to each sample, followed by 30 µl of 2 µg ml⁻¹ pancreatic DNase (Millipore). Digestion was carried out for 4 h at 37 °C and the reaction mixes chilled on ice. Following this 170 µl 10 mg ml⁻¹ bovine serum albumin and 830 µl 7% TCA were added to each sample, followed by mixing and 10–15 min incubation on ice. Acid-insoluble material was removed by centrifugation and the acid-soluble supernatants (containing 65–70% of the total radioactivity) extracted 3 times with 8 volumes of cold diethyl ether. After removal of remaining ether with a stream of dry N₂, 0.01 volume of 1 M Tris, pH 8.2 was added to each sample and the samples stored at 0 °C. A secondary irradiation of the extracts was performed as in the legend to Fig. 1. Determination of thymine-containing pyrimidine dimers was by the method of Goldman and Friedberg³⁹. Determination of photoreleased thymine by thin layer chromatography was on polyethyleneimine-cellulose plates (Brinkman) in *n*-butanol–acetic acid–water 40:6:15.

to oligonucleotides exhibiting a definite photorelease of labelled thymine (Table 2, sample 4). In uninfected *E. coli*, therefore, excision of pyrimidine dimers is apparently not mediated by a pyrimidine dimer-DNA glycosylase.

The fraction of thymine in dimers decreased during the secondary irradiation of the excision products (Table 2, sample 2), while it increased in the DNase digests (Table 2, samples 3 and 4). Chromatography on DEAE-paper indicated that the label in the digests was mainly in species larger than trinucleotides; in contrast, tritium in the excision products was primarily in the form of dTMP, as originally observed in ref. 17. Since 254 nm light catalyses an equilibrium in which less than one-fourth of the adjacent pyrimidines are dimerized¹⁹, and since in these excision products the adjacent pyrimidines are initially largely dimerized, the proportion of dimers is expected to be reduced by the secondary irradiation. On the other hand, the oligonucleotides of the DNase digests are longer and thus would be expected to have a substantial number of non-dimerized adjacent pyrimidines. In this case, therefore, one expects an increase in the dimer content upon additional irradiation.

5,6-Hydrated thymine residues are removed by DNA glycosylase activity of endonuclease III

Exposure of DNA to UV light or other ionizing radiation produces thymine bases saturated in the 5,6-double bond²⁰. Systems for repairing these lesions have been shown to be present in various organisms^{20,21}, including *E. coli*²². An enzyme, endodeoxyribonuclease III, has been isolated from *E. coli* and cleaves DNA which has been heavily UV-irradiated²³. Characterization of extensively purified endonuclease III indicated that it recognizes lesions other than pyrimidine dimers¹². Treatment of DNA with osmium tetroxide rendered it especially susceptible to the enzyme, suggesting that the 5,6-dihydroxydihydrothymine residues so formed are the sites of action of endonuclease III. Remarkably, endonuclease III is also highly active in cleaving partially depurinated DNA¹². A simple hypothesis to explain the varied activities of endonuclease III is that it might contain both DNA glycosylase activity specific for 5,6-hydrated thymine and AP endonuclease activity.

Since UV light produces many lesions in DNA⁴, DNA-glycosylase activity of endonuclease III was tested by analysing the products of reaction of the enzyme with ³H-thymine-labelled DNA treated with osmium tetroxide (Fig. 2). Tritium label was recovered as free 5,6-hydrated thymine in amounts dependent upon the level of oxidation. High pressure liquid chromatographic analysis of a sample after extensive incubation showed the presence of tritium only in positions of authentic 5,6-dihydroxydihydrothymine and to a lesser extent in that of

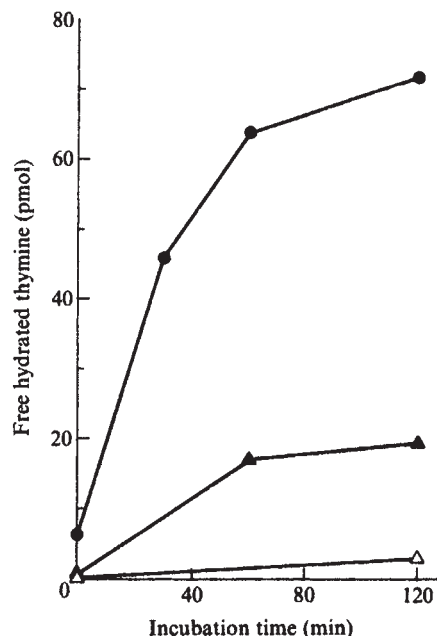


Fig. 2 Release of 5,6-hydrated thymine from OsO₄-oxidized DNA by endonuclease III. Endonuclease III was prepared as described by Gates and Linn¹². DNA was treated with osmium tetroxide by mixing 190 µl T7 ³H DNA (1.35 mM nucleotide, 25,000 c.p.m. nmol⁻¹) with 255 µl 0.3 M potassium phosphate, pH 12.3 and 70 µl 4% aqueous solution of OsO₄ (Polysciences) (circles) or with 190 µl 0.3 M potassium phosphate, pH 12.3 and 5 µl 4% aqueous OsO₄ (solid triangles). After 30 min at room temperature 195 µl 1 M potassium phosphate, pH 4.0 were added and unreacted OsO₄ extracted twice with 20 vol. of cold diethyl ether³⁵. Remaining ether was removed with N₂ and the DNA reannealed as described by Davis and Hyman³⁶. The DNA was then dialysed against 10 mM Tris, pH 7.5 at 4 °C, EDTA was added to 1 mM, and the solution was concentrated under vacuum. The concentrate was dialysed against 10 mM Tris, pH 7.5 and stored frozen at -20 °C for no longer than 2 days before use. Enzyme reaction mixtures contained 20 nmol (circles) or 38 nmol (triangles) of DNA nucleotide in 67 mM Tris, pH 8.2-25 mM KCl-0.005% Triton X-100, with enzyme or buffer added to a final volume of 300 µl. After incubation at 37 °C for the indicated times, samples on ice were precipitated and analysed by HPLC as in Fig. 1 legend, except that carrier 5,6-dihydroxydihydrothymine and 5,6-dihydrothymine (Sigma) were added, and 150 µl fractions collected at 1 ml min⁻¹ flow rate, 730 p.s.i. Water (150 ml) was added to each fraction and the absorbance at 254 nm determined prior to scintillation counting as for Fig. 1. 5,6-dihydroxydihydrothymine was made by OsO₄-treatment of thymine³⁷ (Sigma) or by the method of Cadet and Teoule³⁷. Open triangles, untreated DNA; solid triangles, DNA exposed to 0.054% OsO₄; circles, DNA exposed to 0.52% OsO₄.

5,6-dihydrothymine (Table 3). Furthermore, analysis of the extent of nicking of the DNA by alkaline sucrose gradient sedimentation indicated that the number of endonucleolytic incisions is equal to or somewhat less than the number of base molecules released by the enzyme (Table 3). At early times the formation of nicks seemed to lag behind the release of damaged bases. Thus it seems that endonuclease III acts first by cleaving the glycosylic bond of a 5,6-hydrated thymidylate residue and then by cutting the phosphodiester chain at the resultant apyrimidinic site. It is noteworthy that the 5,6-dihydrothymine did not appear to be formed by OsO₄ treatment (Table 3). Instead, it may have been introduced during the preparation of the DNA or may have been formed during DNA storage.

AP endonucleases associated with T4 UV endonuclease and endonuclease III

Given that purified *M. luteus*²⁴ and T4 UV endonucleases¹³ and *E. coli* endonuclease III each contain both DNA glycosylase and AP endonuclease activities, one may ask whether the two activities in some or all of these preparations are properties of a single enzyme. Alternatively, the presence of two activities in such preparations might be an artefact of purification and assay procedures: since endonucleolytic activity was being used as an assay in each case, AP endonuclease needed to be present in order to monitor *N*-glycosylic base removal as strand breakage.

Table 3 Comparison of base release and nicking by *E. coli* endonuclease III

DNA treatment	Endonuclease incubation (min at 37 °C)	Free hydrated thymine released (pmol)		Nicks present (pmol)
		Dihydroxy-dihydrothymine	Dihydro-thymine*	
None	0	<0.2	<0.2	1.9
	120	<2.1	3.0	3.1
0.054% OsO ₄	0	<2.3	0.2	2.0
	60	16	1.0	11
	120	18	1.3	16
0.52% OsO ₄ †	0	12.8	<0.02	8.3
	60	120	<0.02	63
	120	130	<0.02	>72

Base release was determined by HPLC and OsO₄ treatment was carried out as described in the legend to Fig. 2. Nicking of the DNA was determined as described in the legend to Table 1. Endonuclease reactions were as described in the legend to Fig. 2.

*Assuming specific radioactivity is that of the DNA thymine, not the undiluted label used during cell growth.

†Values are normalized from 0.27 to 0.48 pmol T7 DNA genomes as used in untreated and 0.054% OsO₄ experiments.

However, there is some evidence against this possibility. With endonuclease III, the ratio of UV to AP endonuclease activity is constant during inactivation of the enzyme by both heat and storage, and the reaction optima for both activities are about the same. Similarly, T4 UV endonuclease loses both UV and AP endonuclease activities at the same rate during storage (data not shown). In addition, attempts to separate AP endonuclease from 5,6-hydrated thymine glycosylase activities have failed, and endonuclease III activity is not altered by isoelectric focusing²³. Such studies have not been reported for the UV endonuclease of *M. luteus*; nevertheless, the cleavage products of the AP endonuclease activity of that enzyme are like those of endonuclease III and T4 UV endonuclease; that is, nicks containing a 3'-terminal AP deoxyribose¹³. In contrast, cleavage by endonuclease IV (ref. 13), endonuclease VI (ref. 25) and human placental AP endonuclease²⁶ is on the opposite side of the base-free deoxyribose residues so as to yield nicks containing 5'-terminal deoxyribose-5-phosphate. Thus, the AP endonucleases associated with DNA glycosylase activities are all of the same type and are distinct from those of the AP endonucleases lacking apparent associated DNA glycosylase activity (at least for the limited number characterized to date). It is also possible that the AP endonuclease components of endonuclease III and T4 UV endonuclease are derived from a common source. However, the detergent Triton X-100 stimulates both the AP and UV endonuclease activities of endonuclease III some 5- to 10-fold, but it is without effect on either the AP- or UV-specific endonucleolytic activity present in our preparation of T4 UV endonuclease. In addition, T4 UV endonuclease fails to cleave OsO₄-treated PM2 DNA¹³.

In summary, it is possible that the AP endonuclease activities are not fortuitously associated with their respective *N*-glycosylase components, but instead are companion activities in repair complexes or even single polypeptide chains.

Schemes for UV repair involving DNA glycosylase activities

A scheme for UV repair which extends the base excision studies of Lindahl⁶ is emerging from these and related studies^{10,13} (Fig. 3). A DNA glycosylase activity specific for pyrimidine dimers (T4 or *M. luteus* UV endonuclease) or 5,6-hydrated thymine (endonuclease III) would first hydrolyse the glycosylic bond of the damaged base. For 5,6-hydrated thymine, the base is completely released from the DNA, as is the case with glycosylase removal of uracil⁶, 3-alkyl-adenine⁸, hypoxanthine² and formamidopyrimidine⁹. For pyrimidine dimers, a structure is formed which retains the dimer in the DNA. It is this pyrimidine dimer attached by only a single glycosylic bond which releases a single pyrimidine when exposed to a secondary dose of irradiation. Judging from the results of Minton *et al.*²⁷, photoreactivating enzyme *in vitro* would also be capable of acting to cleave dimers at these nicked sites.

Following glycosylase action, two routes are possible for endonuclease incision: hydrolysis of the bond either on the 5'- or on the 3'-side of the AP site. Cleavage on the 3'-side, as has been shown for preparations of endonuclease III and T4 UV endonuclease¹³, yields a nick containing a 3'-terminal deoxyribose. Such a structure is an ineffective primer for *E. coli* DNA polymerase I (ref. 13), but can be rendered effective by removal of the 3'-terminal AP site. This removal can be carried out by endonuclease IV or endonuclease VI of *E. coli*¹³, resulting in a gap of one nucleotide at the site of damage (Fig. 3). DNA polymerase I can then excise and resynthesize the damaged region, with DNA ligase restoring the continuity of the DNA to complete the repair. Alternatively, initial cleavage at the AP site arising from glycosylase action might be carried out by an AP endonuclease that leaves a 5'-terminal AP deoxyribose and 3'-OH terminus. As shown previously¹³, such nicks directly provide efficient primer termini for *E. coli* DNA polymerase I.

It now seems that in *E. coli*, specific excision repair pathways might proceed via base-excision with specificity for the type of

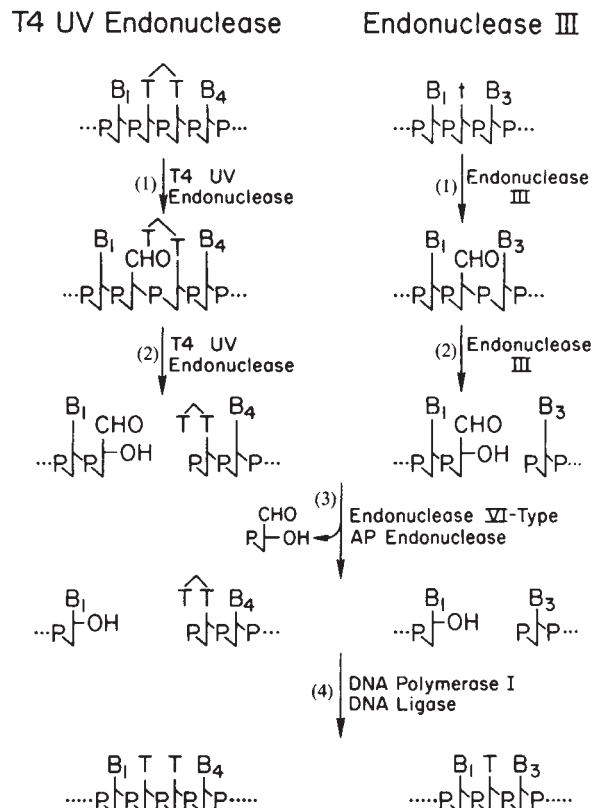


Fig. 3 Excision repair of UV-damaged DNA. The DNA is presumed to be duplex, but for simplicity only one strand is shown. t represents 5,6-hydrated thymine. Endonuclease VI-type refers to AP endonucleases that cleave on the 5'-side of AP sites.

damage lying with DNA glycosylases, while strand incision would occur by virtue of AP endonuclease specificity. Alternatively, apurinic sites might be repaired in a single step by insertion of the appropriate purine base²⁸. Endonuclease incision at damaged nucleotide residues would then occur as a back-up mechanism and for cases where no specific glycosylase is available. Such repair would be mediated by enzymes such as endonuclease V, or the *uvrABC* complex, which appear to recognize many abnormalities in DNA^{5,29}, rather than by the more specific enzymes such as endonuclease III.

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LETTERS

Radio jets and bridges in the classical double sources 3C388 and 0816+526

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We report on 4885-MHz full synthesis high-resolution ($1''$) observations [using the NRAO Very Large Array (VLA)] of the 'classical' double radio sources 0816+526 and 3C388. Classical double sources are characterized by strong emission peaks (lobes) at the extremities of a linear structure. Although at lower resolution these sources both appear to have simple double-lobed morphologies, the VLA maps reveal several important new features. 0816+526 was found to have very sharp leading edges indicative of strong deceleration, possibly as a result of encountering a dense extragalactic background gas. The transverse boundaries of the components are also quite steep but still consistent with constant emissivity-filled cylinders rather than limb-brightening. The morphology of 3C388 is dramatically different from 0816+526, since it includes a bright radio jet. Unlike previously observed radio jets which are usually found between the nucleus of the radio galaxy and its extended lobes, the jet in 3C388 is embedded inside the southwestern lobe and a prominent gap in the radio emission exists between the radio nucleus and the beginning of the jet. These observations clearly illustrate that a variety of source substructures, which must be dealt with in theoretical models, exist within radio galaxies which were previously classified simply as classical doubles.

0816+526 and 3C388 were chosen for detailed study with the VLA because their morphologies as seen in lower-resolution maps are typical of classical doubles. 0816+526 is associated with a galaxy in the direction of the cluster Abell 643 (distance class 5), although there is no redshift information to confirm its membership¹. 3C388 (ref. 2) is identified with a cD galaxy ($z = 0.0917$)³ in the direction of a poor cluster. Both sources are quite luminous with 178-MHz radio powers, P_{178} , of $\geq 3 \times 10^{25} \text{ W Hz}^{-1} \text{ sr}^{-1}$ for 0816+526 (assuming $z \sim 0.14$) and 3C388. Furthermore, both radio galaxies meet Fanaroff and Riley's Class II criteria (that is, most of the radio power emanates from the source extremities)⁴. The VLA maps of these radio galaxies have revealed a variety of structures which are as rich and complex as those seen in the lower luminosity doubles^{5,6}. Several of these newly discovered features raise some interesting theoretical questions.

3C388 and 0816+526 were observed during a 24-h period on 9–10 July 1979 with the VLA. For these observations, a total of 16 antennas were available in the array with baselines ranging from $\sim 60 \text{ m}$ to $\sim 18 \text{ km}$. Although the spacings were distributed non-uniformly (grouped heavily with baselines $\leq 2 \text{ km}$), the

resulting u, v coverage allowed us to adequately map structures at most position angles in these high-declination sources. A bandwidth of 50 MHz centered on the frequency 4885 MHz ($\lambda = 6 \text{ cm}$) was used. The data were collected, edited and calibrated in a fashion similar to that discussed in Owen *et al.*⁷. 'Dirty' maps were constructed and 'cleaned' using the standard techniques.

Contour maps of the total intensity distribution for 0816+526 and 3C388 are shown in Figs 1 and 2, respectively. The shaded ellipses illustrate the FWHM contour of the elliptical clean beams used in restoring the maps.

The crosses in Figs 1 and 2 mark the positions of optical objects in the fields as seen on the Palomar Sky Survey (PSS) E-prints. The sizes of the crosses represent the formal $1''$ – $2''$ error in the optical measurements but they do not reflect the error in locating the galaxy nucleus on the overexposed images of the PSS. It is this second error which is likely to be responsible for the offset of the cross in Fig. 2 from the presumed 'nuclear' radio component in 3C388.

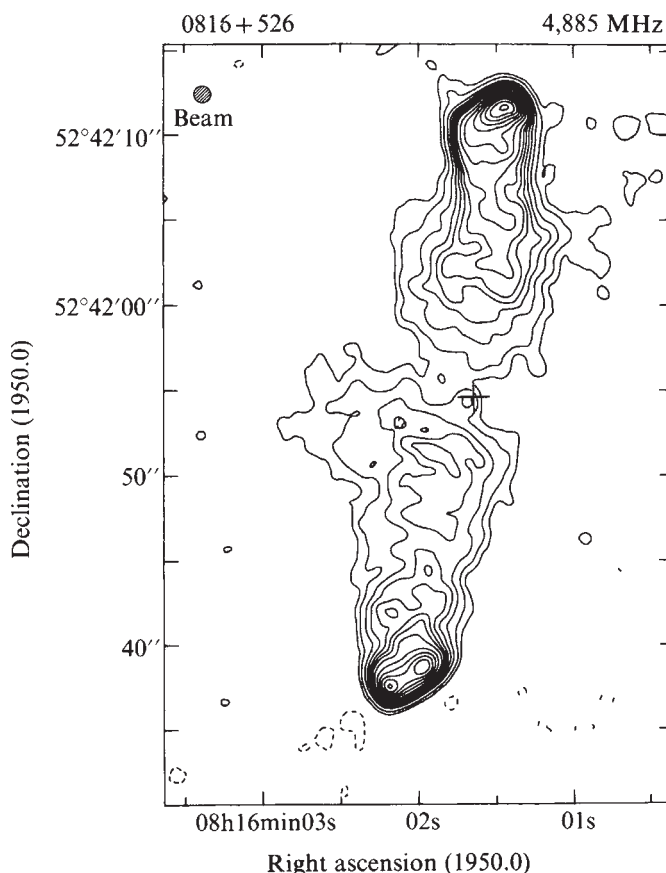


Fig. 1 Total intensity contour map of 0816+526 at 4,885 MHz. The contour levels are 21, 19, 17, 15, 12.6, 10.5, 8.4, 6.3, 5, 4.2, 3.6, 2.5, 1.7, 0.84, 0.42, -0.42 , and -0.84 mJy per clean beam area. The shaded circle represents the half-power contour of the VLA clean beam ($1''$). The cross marks the position of the galaxy identified with 0816+526 as measured from the PSS E-print for this field.

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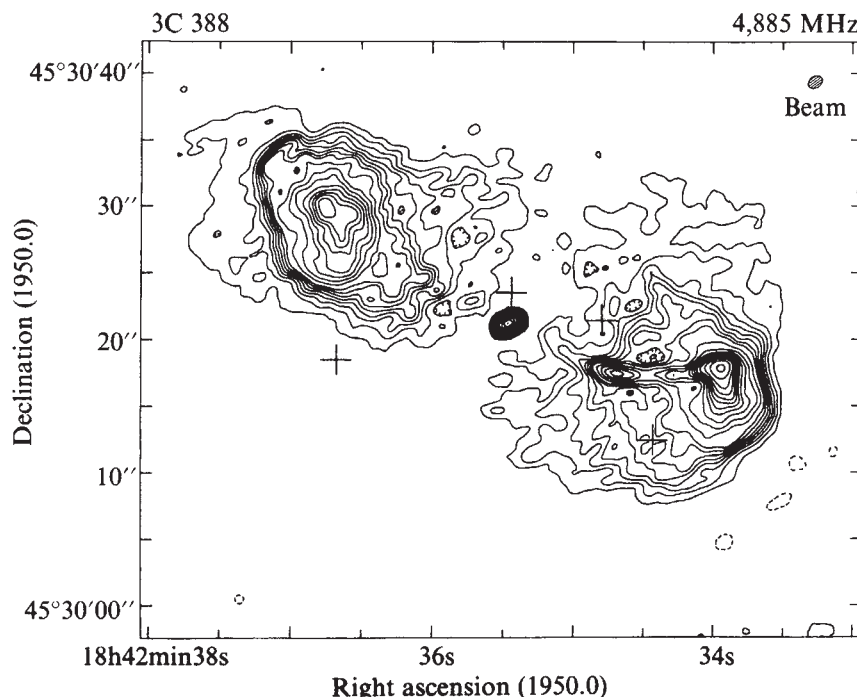


Fig. 2 Total intensity contour map of 3C388 at 4,885 MHz. The contour levels are 62, 50, 37, 25, 19, 13.6, 12.4, 11, 10, 8.7, 7.4, 6.2, 5, 4.3, 3.7, 3.1, 2.5, 1.9, 1.2, 0.6, -0.6, and -1.2 mJy per beam. The peak on this map is 62 mJy. The shaded ellipse represents the half-power contour of the VLA clean beam ($1.2'' \times 0.9''$, p.a. = -61°). The crosses mark the positions of galaxies in the field of this source as measured from the PSS E-print.

The rich variety seen in the fine structures of both of these sources, in conjunction with high resolution observations of other double sources^{6,8-10} indicates that the simple 'classical' double structure previously observed in many radio sources may often be an artifact of insufficient resolution. The new features, such as jets, shells and hot spots which are revealed, may provide the impetus for the testing and development of more detailed theories of radio source evolution. In what follows, since the structures of these two sources are qualitatively different, we shall discuss the structure of each source separately.

Referring to Fig. 1, this source has very sharp leading edges on both the northern and southern extremities. Both of these leading edges are curiously flattened (dense concentration of flux contours) along nearly parallel lines (to within 5°) at position angles of about 57° possibly indicating a large-scale symmetry in the source confinement mechanism.

The northern component of 0816+526 contains a very intense hot spot ($S_6 = 21.2$ mJy per clean beam area) elongated in p.a. 55° , while the southern component contains a pair of hot spots ($S_6 = 17.2$ mJy per beam and $S_6 = 16.8$ mJy per beam) having nearly equal intensities whose axis lies at p.a. 60° . The strong concentration of the flux contours of both components toward the leading edges implies that steep gradients exist in the internal pressures in both components. This might be expected, if both components are strongly decelerated, possibly as a result of encountering an enhanced background density located well outside the parent galaxy. This dense background gas might be attributed to gaseous remnants from previous activity. An encounter with an enhanced density is also consistent with the sudden reductions in the transverse dimensions of each component observed at declinations of $\delta = 52^\circ 41' 50''$ and $\delta = 52^\circ 42' 06''$, respectively. Numerical calculations¹¹ show that these factors of 2 reductions in transverse radii (as measured from second moments of the one-dimensional strip scans)¹² can be induced by encounters with background media whose densities are enhanced by about a factor of 10.

Another feature of 0816+526 which differs from the structures usually observed in extended doubles is the existence of sharp boundaries along the trailing sides of both components. This is especially noticeable along the sides of the northern component. We have investigated the possibility that trailing regions of this source might be 'limb-brightened' (in the sense that the volume emissivity near the outer boundary of the source exceeds the axial emissivity) by comparing brightness distributions along transverse cuts at declinations between $\delta =$

$52^\circ 41' 44''$ and $\delta = 52^\circ 42' 10''$ with the brightness distributions expected from constant emissivity-filled cylinders whose axes are perpendicular to the line of sight. We find no evidence for limb-brightening since none of these cuts have brightness distributions which rise significantly faster than the constant emissivity case. Even the region in the north component with sharp sides ($52^\circ 42' 06'' < \delta < 52^\circ 42' 10''$), the brightness distribution can be adequately described by two parameter (radius, peak brightness) least squares fits with filled cylinders ($0.92 < \text{correlation coefficient} < 0.95$; probability that correlation could result from random data in $< 0.1\%$). As pointed out by De Young¹³, limb-brightening is likely to occur in theoretical models of ram pressure radio sources (whether beams, plasmoids or massive objects) in which relativistic particles are transported and/or accelerated in the sources bow shock. Our failure to detect limb-brightening in this source which has such well-developed hot spots and sharp leading edges seems to indicate that bow shock transport is not likely to be the dominant mechanism for injecting relativistic particles into the trailing regions.

Although the luminosity of 3C388 is roughly comparable to the estimated value for 0816+526, the morphology of this double source is dramatically different as shown by the total intensity map (Fig. 2). Certainly the most interesting feature in this source is the complex jet which is embedded in the south-western lobe. The inclusion of this jet in an extended lobe is remarkable since all radio jets observed previously⁵⁻⁷ have been located between the galactic nucleus and any extended radio lobes which might be present. In 3C388, however, there is a very noticeable gap in the radio emission between the galactic nucleus and the jet. Furthermore, the jet, which runs almost directly east-west, is definitely not parallel to the double source major axis, having an inclination angle of about 30° with respect to this axis.

The jet (average $S_6 \sim 8$ mJy per beam, percentage polarization, $\pi \leq 5\%$) contains two prominent hot spots at opposite extremities (western hot spot: $S_6 = 41.1$ mJy per beam and $\pi \sim 20\%$ polarized, eastern hot spot: $S_6 = 11.2$ mJy per beam and $\pi < 5\%$). The high-intensity contours of the very bright western hot spot are extended toward the south, a direction which is nearly perpendicular to both the jet and the source major axis. Also, extensions in the low-level contours of the eastern hot spot appear to point towards a very intense nuclear radio component ($S_6 = 62$ mJy per beam) located at the centre of the optical galaxy.

The structure of the northeastern lobe of 3C388 contains two interesting substructures. First, this lobe has a sharp 'V'-shaped ridge embedded in an amorphous region of low-level radio emission. (Apex of the 'V' is $\alpha = 18^{\text{h}} 42^{\text{m}} 37.5^{\text{s}}$, $\delta = 45^{\circ}30'34''$.) The ridge is highly polarized ($\pi \sim 25\text{--}30\%$) with a possibly circumferential magnetic field configuration. This polarization structure will be discussed in more detail elsewhere. Second, the rather diffuse hot spot in this component is located well back from the leading edge of the lobe. Both the jet and the 'V'-shaped shell are missing in the 8-GHz map of De Young *et al.*¹⁴, thus raising the possibility that these regions of the source may have steep spectra ($\alpha > 0.8$ where $S_\nu \propto \nu^{-\alpha}$).

Neither of these sources has a structure which appears to be compatible with either of the extremes in the spectrum of ram pressure confined radio source models: namely, simple continuous beam models on the one hand and single injection plasmoid models on the other. Rather, what seems to be called for is some sort of intermittent beam or multiple explosion model; two concepts in which distinctions have become somewhat blurred. A major remaining distinction between these theoretical models does exist in terms of the plasma density assumed to exist inside the observed radio lobes, with the beam models generally invoking a low-density plasma while the plasmoid models invoke high densities.

As mentioned previously, an attractive interpretation for the structure of 0816+526 invokes the penetration of the radio source into an enhanced background density. The intense hot spots in both components of 0816+526 are then attributable to Rayleigh-Taylor instabilities driven by the large deceleration associated with the enhanced background density. In fact, the rapid growth of Rayleigh-Taylor instabilities, along with a general contraction induced by encountering a higher density, may trigger the type of radiative instabilities discussed by Eilek and Caroff¹⁵ and Christiansen *et al.*¹⁶.

For 3C388, physical conditions seem to be quite different. It seems that this source may involve an interaction between an intermittent beam and/or a fresh plasmoid and the boundaries of an evacuated cavity or bubble. The obvious differences in appearance of the major lobes can then be ascribed to the possibility that the two lobes are in different stages of development. That is, in the southwestern component the leading edge of the beam has already impacted on the shell of the cavity creating the observed hot spot. The jet might then be interpreted as being a narrow part of the beam which has not yet reached the shell or perhaps as a reflection jet of plasma which has been focused by and rebounded from the external shell. This latter possibility¹⁷ is particularly intriguing because of the jet's well-collimated structure and lack of polarization.

The northeastern lobe of the source is then interpreted as being in a less well-developed stage in which a new radio component (represented by the diffuse hot spot) is just beginning to make contact with the steep-spectrum shell source. As this plasma rams further into the apex of the shell it should be decelerated and develop a higher surface brightness more closely resembling the structure of the southwestern component.

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Short-term variability of Cyg X-1 and the accretion disk temperature fluctuation

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Recent theoretical models of Cyg X-1 (refs 1–5) assume that to explain the observed time-averaged spectrum the hard X rays are emitted by inverse-Compton mechanism from an optically thin, hot accretion disk around a black hole. Numerous observations (especially in the energy range ≥ 20 keV) have shown that the source intensity is variable on different time scales^{6–17}. Short-term variability on the time scale of ~ 1 s is especially conspicuous because of its stationary nature and the stability of time scale^{6–11}. A balloon observation was carried out to study the variability in the hitherto unknown hard X-ray range (≥ 20 keV). The results of the analysis, together with those of the former rocket observation, suggest that the variation is essentially spectral, implicating that it originates from temperature fluctuation in an accretion disk. Such a model is discussed here in detail.

Although it has been suggested, along with the shot-noise model, that the variability is caused by local hot spots⁹, how such hot spots are generated and decay in an accretion disk with the relevant time scale remains obscure. Moreover, our previous rocket observation (1.5–25 keV) revealed the complete correlation between the variations of different energy bands (that is, the energy independence of the characteristic time scale ~ 1 s) and energy dependence of the relative amplitude of the variation¹¹, which presents difficulty to the simple shot-noise model^{11,18}. To obtain a wideband picture of the variation, I made a balloon observation in the hard (≥ 20 keV) X-ray range with a large effective area ($\sim 3,000$ cm²) detector comprising pressurized xenon gas proportional counters^{19,20}. The balloon flight was carried out on 22 May 1977 in collaboration with the balloon group of ISAS, University of Tokyo.

The coefficient of variation method of analysis^{11,19} was applied to the balloon data to compare it with the rocket observation. The coefficient of variation for a data bin-width t_b , $\eta(t_b)$, is defined as the standard deviation of the variation intrinsic to Cyg X-1 normalized to its average intensity; it is a dimensionless measure of the amplitude of variation, and as a function of t_b it carries information on the characteristic time $\tau(\eta(t_b))$ is constant for $t_b \leq \tau$ and decreases monotonously for $t_b \geq \tau$ as the variation is smoothed out).

The results are shown in Fig. 1 for the balloon data in two energy bands (20–35, 35–68 keV) with the previous rocket data in three energy bands. The characteristic time is about the same (~ 1 s), but there is a striking difference in energy dependence of the amplitude of variation between low (rocket) and high (balloon) energies; for the soft X rays (≤ 25 keV) the lower the energy, the greater the amplitude of variation, while the opposite is true for the hard X rays (≥ 20 keV).

The relation between the variations observed in different experiments at different times is unknown, so there are

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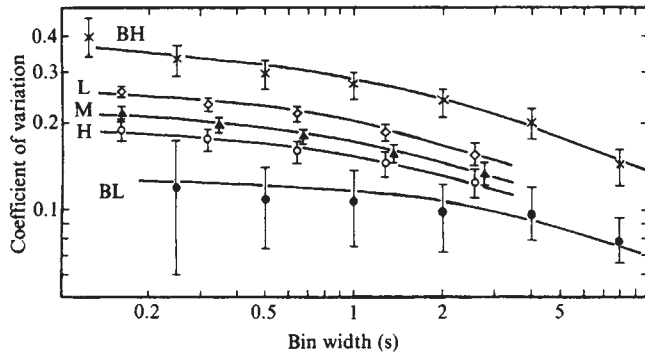


Fig. 1 Coefficients of variation plotted against data bin width for the balloon (BL, 20–35; BH, 35–68 keV) and rocket (L: 1.5–5; M, 5–10; H, 10–25 keV) data.

difficulties when combining the results to make one picture. The difficulty associated with long-term variation may be alleviated by the average flux data in both experiments being consistent with the normal state spectrum. Assuming that the variation is stationary (which is justified at least in $\lesssim 20$ keV range, because it was observed in virtually all the high time-resolution measurements, deriving essentially the same characteristic time scale ~ 1 s) there are three possible interpretations: the soft and hard X-ray variations are (1) positively correlated, (2) statistically independent, or (3) anti-correlated. Although none of these can be ruled out by present observational knowledge, note that the third interpretation (which claims that the variation is essentially spectral, keeping the total X-ray luminosity approximately constant although a narrow-band observation indicates intensity variation, see Fig. 2) is consistently accommodated by the accretion disk model. Spectral variation means that the fluctuation of the electron temperature, and a built-in mechanism of such a fluctuation, does exist in the hot disk proposed in the current models.

Energetics in the disk can be summarized as follows. Gravitational energy of accreting matter is absorbed by ions through viscous dissipation and subsequently transfers to electrons through Coulomb collisions. Electrons are cooled by comptonization of low energy (soft) photons, emitting the bulk of energy as hard X rays. The cooling time scale τ_c ($\sim$ ms) is much shorter than the time scale of Coulomb coupling τ_{ei} ($\propto n^{-1} T_e^{3/2}$), hence the disk is in the two-temperature state^{3,5} ($T_i \gg T_e$; note that $(T_i - T_e)/\tau_{ei} = T_e/\tau_c$ from energy balance). Now if the luminosity of soft photons (L_s) fluctuates, then it will cause the kind of variation suggested from the observations. As the energy supply rate (originally determined by the accretion rate) is considered to be constant ($\sim T_i/\tau_{ei}$) on the time scale of seconds, the energy spectrum has to change to keep the total X-ray luminosity approximately constant. For example, when L_s increases, Compton cooling will lower T_e and a soft (steep) spectrum will result. Stochastic fluctuation of L_s would be

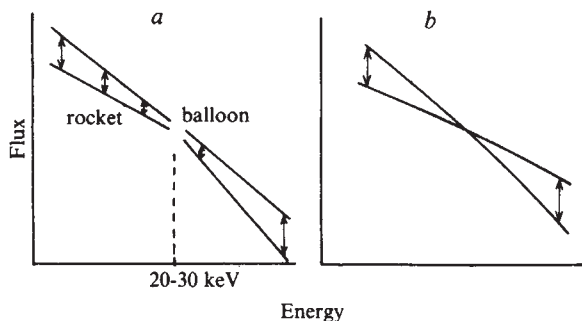


Fig. 2 a, Illustration of the energy dependence of the short-term variation derived from the rocket and balloon observation; b, a tentative interpretation.

expected in the hot disk, which is set up by instabilities and where turbulence is the main source of viscosity.

The simplest description of the variation of T_e is

$$\frac{dT_e}{dt} = \frac{T_i - T_e}{\tau_{ei}} - CL_s(t) \quad (1)$$

where the first and second terms in the right hand side represent Coulomb heating and Compton cooling respectively. (The cooling effect is assumed to be proportional to L_s for simplicity; thus $C\langle L_s(t) \rangle = \langle T_e \rangle / \tau_c$ to meet the steady state solution.) Equation

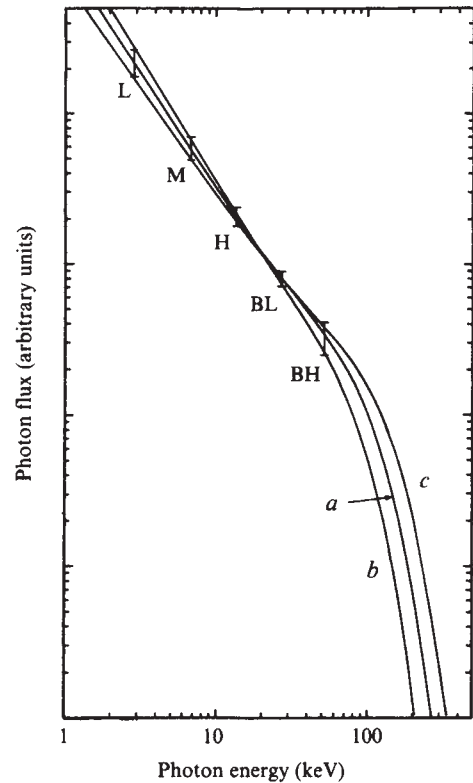


Fig. 3 Theoretical comptonization spectra for a, the best-fit parameters $kT_e = 27$ keV and Thomson optical thickness $= 5$; b, $kT_e = 21$ keV; and c, $kT_e = 33$ keV, normalized to the total X-ray luminosity (1–300 keV). Vertical bars labelled L, M, H, BL, and BH show ± 1 standard deviation of the variations derived from the coefficients of variation at bin-width of 0.5 s (Fig. 1). Each bar is placed at the mean energy of corresponding energy band.

(1) is a Langevin equation¹⁸ for stochastic variable $L_s(t)$. Assuming that $L_s(t)$ fluctuates with a time scale τ_s , so that its power spectrum $I_s(\omega)$ is in the form

$$I_s(\omega) \propto \frac{1/\tau_s}{\omega^2 + 1/\tau_s^2} \quad (2)$$

The autocorrelation function of $T_e(t)$ is then,

$$\langle T_e(t) T_e(t + \tau) \rangle \propto \frac{\tau_{ei} \exp(-|\tau|/\tau_{ei}) - \tau_s \exp(-|\tau|/\tau_s)}{(\tau_{ei} - \tau_s)} \quad (3)$$

Note that τ_{ei} is the only long (~ 1 s) time scale in the inner hot disk, which reflects the high temperature state ($T_i \sim 10^{11}$, $T_e \sim 10^9$ K). τ_s is expected to be much shorter as L_s is considered to originate from cold and dense clumps in the hot disk, where relevant characteristic times (keplerian and hydrodynamic ones) are of the order of milliseconds (ref. 5). Then, equation (3) indicates that the time scale of the variation of T_e (and of X rays) is ~ 1 s just as observed.

Recently Sunyaev and Trümper²¹ have compared the new observational and theoretical results to derive the best-fit disk parameters including a much smaller T_e ($kT_e = 27$ keV) than previously believed. Their theory is based on the (time-averaged) hard X-ray spectrum with high statistical accuracy

obtained from balloon observations by the AIT/MPI group and the analytical solution for the Comptonization spectrum from a spherical isothermal electron cloud. Their results give more semi-quantitative support to the present model. Figure 3 shows the theoretical spectrum for the best-fit parameters ($kT_e = 27$ keV, and Thomson optical thickness = 5) and the spectra for T_e a little ($\sim 20\%$) higher and lower, normalized to the total X-ray luminosity. The node of the flux variation is indeed at 20–30 keV as suggested from the observation. Figure 3 also shows the ± 1 standard deviation of the variations for various energy bands derived from the observational data (Fig. 1) for comparison.

The present model explains the stochastic nature of variability, its characteristic time scale and spectral features at the same time in the context of the conventional accretion disk model for Cyg X-1. Furthermore I suggest that the complex variability of Cyg X-1 over a wide span of time scale may be classified into three categories: (1) non-stationary long-term variation on the time scales from ~ 10 s (ref. 17) to $\sim 10^6$ s (high-low transition^{12–15}) due to fluctuations in the outer cool disk and changes in accretion rate⁵; (2) stationary short-term variation discussed here; and (3) millisecond pulsation¹⁶ which presumably reflects the intrinsic time scale to the inner hot disk. A more refined version of extended-range, high time-resolution observation will clarify the nature of the variability.

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The 20- μ m brightness temperature of the unilluminated side of Saturn's rings

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The 20- μ m brightness temperature of the unilluminated (north) side of Saturn's rings is reported here to be 56 ± 1 K. This measurement, together with previous measurements of the rings at 20 μ m, shows that the brightness temperature of the B ring can be approximated by the simple equation $T_B^4 = 56^4 + 1.52 \times 10^8 \sin^2 |B'|$, where B' is the saturnicentric declination of the Sun. We have assumed that emission from the Cassini division is negligible. The constants in this equation may be understood in terms of a two-component heating model for the ring particles—sunlight and radiation from Saturn. We discuss the implications of the IR data for several published ring models.

Table 1 19.8 μ m photometry

Ring position flux	$5.75 \pm 0.22 \times 10^{-19} \text{ W cm}^{-2} \mu\text{m}^{-1}$
Sky position flux	$3.57 \pm 0.18 \times 10^{-19} \text{ W cm}^{-2} \mu\text{m}^{-1}$
Net ring flux	$2.18 \pm 0.40 \times 10^{-19} \text{ W cm}^{-2} \mu\text{m}^{-1}$
Ring brightness	$5.88 \pm 1.08 \times 10^{-9} \text{ W cm}^{-2} \mu\text{m}^{-1} \text{ sr}^{-1}$
Corrected ring brightness*	$9.06 \pm 1.66 \times 10^{-9} \text{ W cm}^{-2} \mu\text{m}^{-1} \text{ sr}^{-1}$
Ring brightness temperature	$56.0 \pm 0.9 \text{ K}$

*See text.

Previous observations showed that the brightness temperature of Saturn's B ring gradually decreased as B' diminished. The current time period is critical because observations at the small values of B' can most effectively distinguish between competing models for the B ring, as shown by Nolt *et al.*¹. In addition, for several months near the beginning of 1980 the unilluminated side of the rings could be observed from the Earth. This opportunity arises whenever the Earth and the Sun are at opposite sides of the plane of the rings, as can occur during the ring plane crossing². The first IR observations of the unilluminated side of the rings were made with the Pioneer 11 spacecraft, which found a 45- μ m brightness temperature of ~ 55 K (ref. 3). [Ingersoll (personal communication) now considers the Pioneer 11 observation of the unilluminated side of the rings to be a firm detection rather than an upper limit.]

The observations were made with the 3-m IR Telescope Facility (IRTF) at Mauna Kea on Hawaii on 6 January 1980 (UT). A newly developed 2–30- μ m liquid helium cooled bolometer system was used for the observations. For the ring observations, a 2-arc s aperture was used with a cooled 19.8- μ m filter ($\Delta\lambda = 6 \mu\text{m}$) which defined the wavelength interval. The aperture was centred on the B ring ansa, but the small opening of the rings (the maximum extent of the minor axis was 1.7 arc s) prevented the separation of the A and B rings. The C ring was completely out of our aperture.

The centre of the B ring ansa was only 6 arc s from the edge of the disk of Saturn, and as a result scattered and diffracted radiation from the disk produced a spurious signal (which we call 'sky' signal). We have measured the sky signal at the same radial distance from the limb as the rings but displaced 4 arc s north and south. The signal at the ring position and the adjacent sky is shown in Table 1 (the north and south sky measurements have been combined). As there was no significant difference in the signals at the ring position between the A and B ring ansae, the signals at both ansae have also been combined in Table 1.

The difference in measured flux between the ring and sky positions was $2.18 \pm 0.40 \times 10^{-19} \text{ W cm}^{-2} \mu\text{m}^{-1}$. The quoted error is the sum of the errors of the ring and sky positions and is therefore a conservative estimate. The solid angle of the rings within our aperture was determined graphically to be $3.71 \times 10^{-11} \text{ sr}$, using previously published constants⁴, so that the surface brightness of the rings is $5.88 \pm 1.08 \times 10^{-9} \text{ W cm}^{-2} \mu\text{m}^{-1} \text{ sr}^{-1}$.

The first dark ring of the Airy diffraction pattern of the telescope has a diameter of 3.4 arc s. Therefore, some of the flux from the ring was diffracted out of the beam. From a two-dimensional convolution of the diffracted ring with our aperture, we estimate that approximately 35 $\pm$ 5% of the ring flux was diffracted out of the beam. After adjusting for this we obtain a corrected ring brightness of $9.1 \pm 1.7 \times 10^{-9} \text{ W cm}^{-2} \mu\text{m}^{-1} \text{ sr}^{-1}$, which corresponds to a brightness temperature of 56 ± 1 K at 19.8 μ m. This brightness temperature agrees very well with that measured by Pioneer 11 at 45 μ m.

It is difficult to correct our result for the emission from the Cassini division because its optical depth and brightness temperature are both very uncertain. The normal optical depth has recently been estimated to be in the range 0.01–0.05 (refs 5–7). For this range of optical depth, the brightness temperature of the Cassini division must be less than 64–71 K for the Cassini division to emit less than 10% of the observed emission. As Nolt *et al.*¹ found that the C ring brightness temperature was 82–85 K at $B' = -6.5^\circ$, a brightness temperature for the Cassini division

Table 2 Previous observations of the B ring of Saturn

Saturnicentric solar declination $ B' $	B ring brightness temperature at 20 μm (K)	Ref.
26.3	94 ± 2	16
26.4	96 ± 3	17
25.8	90.4 ± 1.5	18
16.3	84 ± 2	10
11.8	77.5 ± 3	1
11.5	79.4	19*
6.5	74 ± 3	1
2.83	60–70	3†

*This is based on the higher flux calibration adopted by Sinton *et al.*¹⁹

†Range of 45- μm brightness temperature from Pioneer 11.

of ≤ 70 K at the time of our observations ($B' = -0.881^\circ$) is plausible. In the following we assume that the emission of the Cassini division is negligible, pending more precise estimates of the optical depth and brightness temperature of the Cassini division.

The ring brightness temperature must be very close to the actual temperature of the unilluminated side of the rings, as the rings are opaque in the line of sight. From the B ring normal optical depth of ≈ 1 at visual wavelengths⁸, we estimate the visual line of sight transmission in the direction of the Earth to be $1/\exp(1/\sin 1.72^\circ) \approx 10^{-15}$, where 1.72° is the saturnicentric declination of the Earth. Therefore, with unit emissivity, the physical and brightness temperatures would be identical. For all plausible emissivities, the difference will be very small. For example, at 20 μm , an emissivity of 0.8 would result in an underestimate of the physical temperature of less than 1 K.

We further suggest that our result for the dark side provides a measure of the A and B ring brightness temperature in the absence of solar heating, the condition achieved when $B' = 0^\circ$. Direct solar heating certainly does not influence our observation, as the line of sight transmission for sunlight is even less than the transmission as viewed from the Earth. Heating of the dark side by scattered sunlight is possible⁹, but we ignore it for the present. A qualitative explanation of the observed ring brightness of the unilluminated side as discussed below does not require additional heating by scattering.

Our measurement is shown in Fig. 1 along with previous 20- μm observations of the B ring that are listed in Table 2. No correction for the distance of Saturn from the Sun has been applied. Such a correction, as applied by Nolt *et al.*¹⁰, results in a change of brightness temperature of ≤ 1 K, which is less than the uncertainty in the measurements. A very good fit to the data can be obtained using the empirical formula

$$T_B^4 = T_0^4 + C \sin |B'| \quad (1)$$

where T_0 and C are constants. The first term on the right accounts for the heating from the disk of Saturn that results from reflected solar radiation and IR emission from Saturn. The second term in equation (1) accounts for solar heating as a function of B' , a parameterization which has been suggested previously by Murphy¹¹ and by Pollack *et al.*¹² for various simplified ring models.

Our observations give $T_0 = 56$ K because this is the expected value of the B ring temperature for $B' = 0$ as discussed above. We have neglected here any difference in the A and B ring temperature, because we do not have sufficient information for a two-parameter fit. We find that a value of $C = 1.52 \pm 0.17 \times 10^8 \text{ K}^4$ gives the best fit to the data, as shown in Fig. 1.

Can the ring temperature of 56 K be explained only from heating by the disk of Saturn? We assume that an idealized ring particle is illuminated by Saturn on only half of its northern hemisphere. Radiation from Saturn from the other side of the ring plane is assumed to be blocked by other ring particles. We then obtain the following simple radiation balance equation

$$(1-A)f = 4\alpha\epsilon\sigma T_0^4 \quad (2)$$

where A is the Bond albedo, f is incident flux from Saturn (W m^{-2}), ϵ is the particle emissivity, σ the Stefan-Boltzmann constant and T_0 the physical temperature of the ring particle. α is a constant which is 1 if the particle radiates into 2π sr and 2 if the particle radiates into 4π sr. The Bond albedo is very uncertain, but a value of 0.6 seems reasonable¹³.

For $A = 0.6$ and $T_0 = 56$ K, we obtain from equation (2) the following expression for the incident flux from Saturn

$$f = 5.58 \alpha \epsilon \text{ W m}^{-2} \quad (3)$$

This can be compared with the expected incident flux at the centre of the B ring resulting from the reflected solar radiation from the disk of Saturn and the IR radiation of Saturn. Assuming an effective temperature of 94.4 K (ref. 3), we find that the incident flux at the centre of the B ring to be 1.4 W m^{-2} . In addition, for an albedo of Saturn's disk of 0.45 (refs 3, 14), we find that the reflected solar radiation (into 2π sr) contributes an additional 1.1 W m^{-2} . The total amount of incident flux is therefore 2.5 W m^{-2} . This is in good agreement with equation (3) if $\alpha\epsilon \approx 0.5$. Both α and ϵ are unknown, but for $\epsilon = 0.5 \rightarrow 1.0$ —a possible range of emissivity—the particle may radiate into $\approx \pi \rightarrow 2\pi$ sr ($\alpha \approx 0.5 \rightarrow 1$) in this simple model. We conclude that the measured brightness temperature of 56 K can be understood qualitatively in the context of single particle radiation balance. The more realistic multi-particle, multilayer model is discussed below.

The second term in equation (1) can also be understood in terms of a simple model. Following Pollack *et al.*¹², the radiation balance of the rings with sunlight is

$$(131.8 \text{ K})^4 \sin |B'| (1 - A - T_r) = \alpha \epsilon T^4 \quad (4)$$

where T_r is the transmissivity of the rings and T is the ring physical temperature due to solar heating. B' , A , ϵ and α have the same definitions as before. The constant 131.8 K is the temperature of a black disk in equilibrium with the solar radiation at Saturn's distance from the Sun. As discussed above, the transmissivity of the rings is negligible for small B' . Assuming $A = 0.6$ and $T_r = 0$, we obtain from equation (4)

$$T = \frac{1.21 \times 10^8}{\alpha \epsilon} \sin |B'| \text{ K}^4 \quad (5)$$

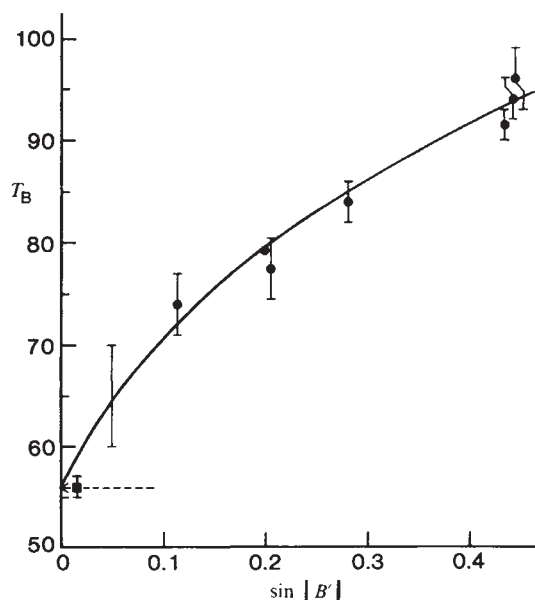


Fig. 1 B ring brightness temperature at 20 μm as a function of $\sin B'$. The solid curve shows the fit achieved with the expression $T_B^4 = 56^4 + 1.52 \times 10^8 \sin |B'|$. The dashed arrow indicates that our measurement of the unilluminated side at $\sin |B'| = 0.015$ is assumed to represent the ring brightness temperature for $B' = 0$, as discussed in the text. All other points are taken from Table 2 and refer to observations of the side of the rings which is illuminated directly by the Sun.

Comparing this expression with the second term in equation (1) with the best-fitting value for the constant C , we see that a reasonable agreement is achieved if $\alpha e \approx 0.8$. Note that the fit is not strictly valid for $B' = 26^\circ$ where $T_r \approx 0.1$.

It is encouraging that roughly the same value for the product αe is obtained in two cases. This increases the possibility that the various parameters have been estimated reasonably well above. However, there are so many areas of uncertainty that we cannot preclude the possibility of fortuitous cancellation for more than one inaccuracy.

The most sophisticated ring model with a prediction for the thermal emission is that by Kawata and Irvine¹⁵. As shown by Nolt *et al.*¹, this model systematically predicts higher brightness temperatures than observed for $\sin |B'| \sim 0.2$. In addition, this model predicts that $T_0 \sim 50$ K, lower than our observed value. Nolt *et al.*¹ also present the predicted brightness temperature for a two-component model of the rings which fitted the data for $\sin |B'| > 0.1$. This model also fails because it predicted T_0 to be ~ 69 K.

In conclusion, the ring IR brightness temperature on the unilluminated side of the rings has been found to be 56 ± 1 K. The decrease of the B ring brightness temperature is apparently proportional to $\sin |B'|$. These characteristics are not explained by any published detailed ring model, but it can be understood on the basis of single particle radiation balance considerations. These results need to be re-examined if the optical depth and brightness temperature of the Cassini division are found to be outside the limits assumed here.

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Pleistocene bimodal response of Antarctic ice

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Extent and volume of the Antarctic ice sheet have important roles in modulating global atmospheric and oceanographic processes and have significant implications for world sea levels¹. The history of ice sheet variations in Antarctica has mainly been founded on studies in the ice free (Dry) valleys of southern Victoria Land (Fig. 1). A detailed glacial chronology by Denton and co-workers^{2,4} is based on the premise that Taylor Glacier and neighbouring Wright Upper Glacier are outlets of the East Antarctic ice sheet and that, "the extent of Taylor Glacier reflects the height of this ice sheet, and, since it terminates on land, records of its former positions provide one of the few indications of former heights of the ice sheet"⁵. That the East Antarctic ice sheet reached its maximum dimensions during each of the last four world-wide interglacials is an important conclusion of this work². New geophysical data from Taylor Glacier and the adjacent ice sheet in Victoria Land, Antarctica are presented here which challenge these suppositions.

Between 1967 and 1978 six seasons of airborne geophysical sounding have completed an extensive network of flight lines over East Antarctica⁶⁻⁸. Ice thickness profiling has enabled compilation of detailed contour maps of the ice sheet surface to an accuracy⁹⁻¹¹ of ± 30 m. Flow lines orthogonal to the contours¹² have been constructed (Fig. 2). In 1974-75 a closely spaced series of Radio Echo Sounding (RES) flight lines was completed over the ice sheet inland of the Dry Valleys (Fig. 3)¹³. These two surveys have defined the pattern of ice flow out of central East Antarctica through the Transantarctic Mountains into the Ross Sea embayment.

In the sector inland of Victoria Land the ice sheet exhibits a wide and prominent ridge which drives ice into the clearly

marked catchments of David and Mulock-Byrd Glaciers (Fig. 2). Within 100 km of the western edge of the Transantarctic Mountains, just inland of Taylor Glacier, the ridge terminates in a small dome. The ice sheet drops by more than 100 m into a saddle between this dome and the main part of the ridge. Altitudes determined by barometry along a single traverse line ~ 30 km south of the dome by Cray during the IGY intimated at a similar elevation distribution¹⁴. Calkin¹⁵, using less reliable radar results, also suggested the possibility of a dome in this vicinity.

RES surface contours establish a flow pattern in which the now well-defined dome and ridge system effectively blocks ice

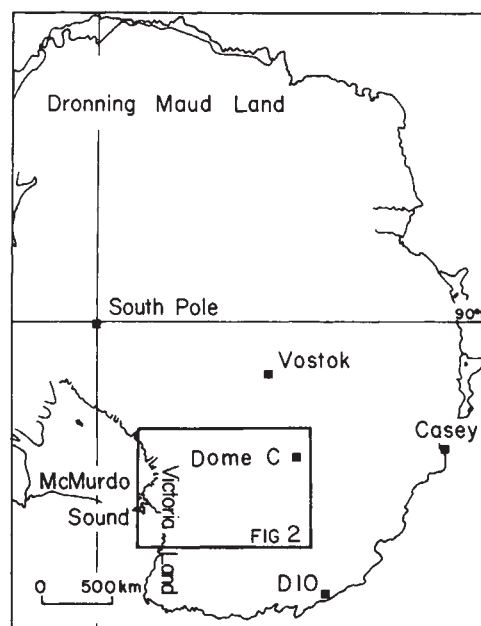
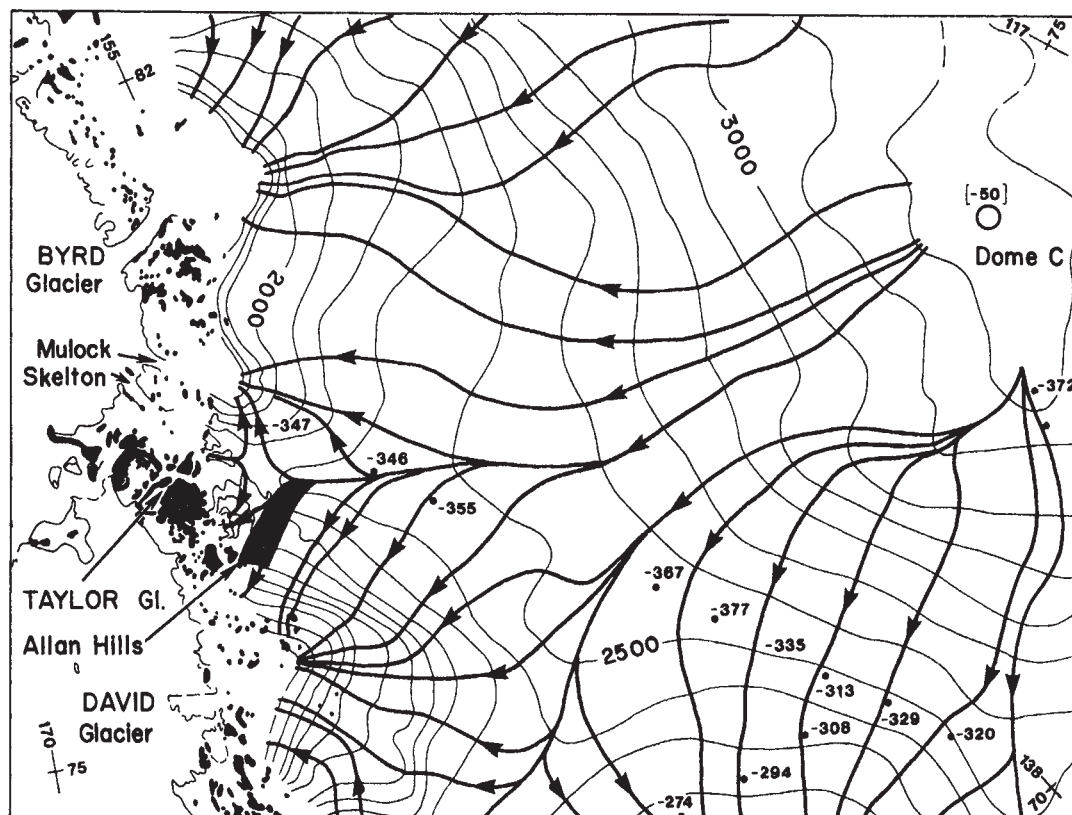


Fig. 1 Antarctic location map (principal places mentioned in text and Fig. 2).

Fig. 2 Ice surface contours and derived flow lines for part of the East Antarctic ice sheet, based on radio echo sounding data^{10,11}. Note diverging ice flow from dome inland of Taylor Glacier. Catchment area of ice flowing towards Allan Hills meteorite site is shown shaded. Selected $\delta^2\text{H}$ and ^{18}O values for surface accumulation are shown^{31-33,38}.



penetration into the Dry Valleys from central regions of East Antarctica. The catchments, therefore, of small outlet glaciers in southern Victoria Land (for example, Ferrar, Wright Upper, Victoria Upper, Mackay and possibly Skelton) are severely restricted and that of Taylor Glacier is less than 5,000 km².

RES results suggest only a local origin for ice in Taylor and adjacent glaciers. To test this hypothesis ice provenance studies, using stable isotope techniques, were performed in January 1978 and January 1979¹⁶.

Five horizontal 1-m ice core samples were taken from the lowermost 1.5 m of exposed ice at the snout of Taylor Glacier in 1978 (Fig. 3). The following season an ice core, 4 m long, was extracted close to the summit of the dome inland of the glacier (Fig. 3). The average accumulation rate in this area is 140 kg m⁻²yr⁻¹ and average snow density in the upper 4 m is 437 kg m⁻³ (refs 14, 17). The core thus sampled 12 yr precipitation and the resulting mean isotopic values are considered representative of surface accumulation at Taylor dome.

Stable isotope results are given in Table 1. Values of $\delta^2\text{H}$ at Taylor Glacier snout range between -327‰ and -341‰ (the lowermost sample), bracketing similar determinations by other workers¹⁸. At Taylor dome (the ice divide) $\delta^2\text{H}$ measurements lie between -341 and -344‰. They correspond closely with those to be expected theoretically¹⁹ given mean annual surface temperature (-43 °C) and elevation (2,400 m). Table 1 also lists isotopic values for ice accumulating in the central parts of East Antarctica. The ice here has a significantly lighter isotopic signature than from southern Victoria Land.

The equivalence of $\delta^2\text{H}$ ratios at Taylor Glacier snout and at the dome (the difference between the two sets of measurements (3‰) is negligible) strongly suggests that the glacier has been in near isotopic equilibrium for the lifetime of oldest ice sampled, and that the preceding conclusions are valid for several thousand years. Integration with respect to time of maximum surface velocities measured by New Zealand workers²⁰ gives an estimated minimum period of ice throughput from dome to snout of 10.5 kyr. Corrections applied to particle paths through Taylor Glacier for zones of basal sliding²⁰ and reduction of velocity with depth²¹ give a more realistic throughput time of 15–16 kyr. If

these dates are approximately correct we attribute the absence of more isotopically negative ice at the base of Taylor Glacier snout (representing the end of Wisconsin cooling) principally to basal melting upstream. Although ice transit calculations are based on the steady-state assumption they, nevertheless, corroborate glacial geological³ and geochemical^{5,22} conclusions that Taylor Glacier did not expand appreciably during the Wisconsin maximum, and provide no evidence for intrusion of 'cold' East Antarctic ice. Post-Wisconsin advance reported by Denton is attributed to increased local precipitation.

The discovery of meteorites²³ on bare ice at the edge of the Transantarctic Mountains at Allan Hills (100 km north of Taylor Glacier dome) also corroborates the notion of ice sheet stability in southern Victoria Land (Fig. 2). The meteorites have ages between 30 and 800 kyr^{24,39} and have been concentrated at

Table 1 Isotopic results from Victoria Land and adjacent ice sheet, Antarctica

Location	Elevation (m)	‰ $\delta^{18}\text{O}$	SMOW $\delta^2\text{H}$	Ref.
Taylor Glacier snout (77°43.6' S 162°16.5' E)				
Lowermost ice 20 cm above bed	NA	<u>-42.8</u>	-341.3	This work
Mean of lowermost 1.5 m snout ice		<u>-41.6</u>	-332.0	This work
		-42.5	-330.0	18
Taylor Glacier dome (77°44' S 158°22' E)	2,400	-43.4	-342.6	This work
Victoria Land plateau				
78°02' S 154°06' E	2,280	<u>-43.5</u>	-347.4	38
77°02' S 152°19' E	2,440	<u>-43.3</u>	-345.5	38
Dome 'C' (74°39' S 124°10' E)	3,240	-50.0	<u>-401.0</u>	33
Vostok (78°27' S 106°48' E)	3,490	<u>-51.4</u>	-412.0	38
South Pole	2,825	<u>-49.4</u>	-396.0	38

Underlined values determined from $\delta^{18}\text{O} = 0.12\delta^2\text{H} + 1.26$ obtained from 58 ice and snow analyses from Victoria Land.



Fig. 3 Taylor and neighbouring glaciers in southern Victoria Land (W, Wright Upper; M, Meserve). Ice sample sites for isotopic analyses: □, 1978; △, 1979. ◇, Oversnow traverse stations¹⁴. Dome defines limit of Taylor Glacier catchment. Elevation contours are in 10^2 m (after ref. 13).

this locality by flow from the surrounding ice sheet and surface ablation²⁵. As Allan Hills presents only a low topographical barrier to the ice sheet any significant thickening in this area (>100 m) would effectively flush-out the meteorites into Mawson Glacier. Assuming that the meteorite infall rate in polar areas is $\sim 1 \times 10^{-6} \text{ km}^{-2} \text{ yr}^{-1}$ (refs 26, 27, 40) we calculate for that part of the ice sheet draining towards Allan Hills ($\sim 1,500\text{--}3,000 \text{ km}^2$, Fig. 2) the time to accumulate the ~ 50 'parent' meteorite new finds²³ is 17–33 kyr. We conclude that the ice sheet in the vicinity of Allan Hills cannot have thickened substantially during this period.

The pattern of fluctuations exhibited by local ice in Victoria Land during the last and possibly earlier glacials is out-of-phase with the bulk of the East Antarctic ice sheet where seaward expansion of continental ice during full glacial periods has been established^{28–33}. We explain antiphase behaviour as a result of differing glacio-climatic controls. On the high plateau accumulation is relatively low ($50\text{--}100 \text{ kg m}^{-2} \text{ yr}^{-1}$)¹⁷ due to steady inland water-vapour depletion of precipitation-bearing air masses that penetrate the continent³⁴. In contrast snow-fall reaching Taylor Glacier and neighbouring ice masses comes primarily from air advected over the Ross Sea, regulated by seasonal ice cover, degree of open water, cyclogenesis and orographic effects^{35,36}.

I suggest that during glacial episodes Northern Hemisphere ice build-up results in a major depression of global sea level causing a modest expansion and thickening of the 'stable' East Antarctic ice sheet. Ice in West Antarctica, however, grounded below sea level, expands dramatically across the Ross Sea continental shelf^{28,37}: the zone of seasonal pack ice and open water is moved northwards by several hundred kilometres, reducing precipitation to those glaciers with limited catchments. Stability or even recession of the ice sheet margin in Victoria Land ensues.

During Northern Hemisphere interglacials rising sea level will trigger inland retreat of the ice sheet. With incursion of open water into the Ross Sea embayment, however, enhanced water vapour flux over Victoria Land results in modest expansion of the ice sheet edge and associated minor glaciers.

Widespread use of the detailed glacial chronology of the Dry Valleys area requires caution. The relationship of local 'outlet' glaciers to the glaciological regime of the East Antarctic ice sheet is clearly complex and often independent. Such conclusions may apply to similar locations along the inland edge of the Transantarctic Mountains (for example, Queen Maud Mountains and Reedy Glacier areas). Sites for future investigation of continental glacial fluctuations in the Transantarctic and other mountainous areas will require careful assessment and interpretation. The most suitable may be the margins of the premier outlet glaciers whose catchments lie deep within the ice sheet (for example, David, Byrd, Jutulstraumen, Shirase, Lambert and Minnesota Glaciers).

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Sublithospheric upwelling distribution

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Hot spots have been defined¹ as centres of volcanism not associated with plate boundaries. Some authors^{2,3} have regarded them as surface expressions of upwellings of relatively high temperature material in the mantle. These upwellings, either deep mantle plumes^{2,4}, or shallow convection cells⁵, create a hot spot on the surface of the Earth only where they have molten and broken their way to the surface. I suggest here that there is a uniform sublithospheric upwelling distribution, and that the ability of upwellings to make themselves evident on the surface, and, therefore, the difference in surface hot spot density among the various plates, depends on the velocity and thickness of each lithospheric plate. From these characteristics and the rate of magma entry into the lithosphere, a normalization of the areal hot spot density can be made to calculate the distance between sublithospheric upwellings.

The major effect of the motion of a plate over a mantle upwelling is to limit the time available for the transfer of heat through the lithosphere. Although the process of lithospheric thinning operates on moving plates^{6,7}, only a fraction of the sublithospheric upwellings are able to penetrate to the surface.

Lithospheric thickness determines the amount of time necessary for an upwelling to penetrate the lithosphere. The thickness depends primarily on the dynamic history of the plate involved, and in particular, its age, which for oceanic regions is related to the distance from the spreading centre^{8,17}.

The rate of intrusion is important in thinning the lithosphere. This determines the rate of ascent of the lithosphere-aesthenosphere boundary.

The sublithospheric upwelling density (expressed as area per upwelling) can be calculated from the above parameters for each plate using the following equation:

$$K = \frac{A}{S \exp(VT/R)} \quad (1)$$

where K is the sublithospheric area per upwelling, A is the area of a particular plate, S is the number of observed hot spots on the plate, V is the velocity of the plate with respect to a fixed mesospheric frame, $T = t/g$ is the thickness of the plate divided by the global average lithospheric thickness, and R is the rate of ascent of the lithosphere-aesthenosphere boundary.

The values of the above parameters are listed in Table 1. The number of hot spots on each plate is as observed by Burke and Wilson¹. Hot spots at or very near plate boundaries were omitted, however, because it would be unclear to which plate they belong, and because the lithosphere becomes very thin at rifts and mid-ocean ridges, giving a great penetration advantage to any upwellings underneath. Although other hot spot lists exist¹⁸, this one was chosen as it is the most comprehensive and adheres most closely to the definition of hot spots used here. The velocities of the plates are as calculated by Minster and Jordan^{19,20}, and agree with other studies^{21,22}. Plate thicknesses, as calculated from heat flow considerations of Sclater *et al.*⁸ have been averaged over the area of each plate. These average values are in agreement with those calculated from other models^{9-14,16}. The global average lithospheric thickness used was 120 km. The rate of ascent of the lithosphere-aesthenosphere boundary is as calculated by Withjack⁶ as $2 \times 10^{-8} \text{ cm s}^{-1}$, corresponding to basaltic magma, assumed constant throughout the Earth as hot spots are generally basaltic¹ worldwide. (If all of the magma entering the lithosphere is extruded²³ or otherwise accounted for²⁴, the basalt represents the bulk composition of the magma,

and the above rate is to be used. If, however, the basalt does not represent the bulk composition of the magma, but is only a portion of the magma which is preferentially pushed through the lithosphere, R is greater, $1.5 \times 10^{-7} \text{ cm s}^{-1}$ for 5% basalt⁶.) It is assumed here that regardless of the value chosen (or any intermediate), the average over the area of each plate remains constant throughout the Earth, and that this value will affect the normalization of the average distance between hot spots, $K^{1/2}$, but not the relative values of K for the various plates.

There are three anomalous plates. The Cocos plate is very small, and has one hot spot, making its hot spot density extremely high. However, so small a plate cannot present a statistically valid distribution, and so can be discounted. The Arabian plate is also very small and has several hot spots which may be associated with the rift system, and as such, cannot be used in this consideration.

The African plate has a large number of hot spots (31), and some authors^{1,3,25} have claimed that it has been stationary with respect to the mantle convection pattern for the past 25 Myr. Minster *et al.*^{19,20} have found the African plate to be moving only slowly about a rotation pole located within the plate. This rotation about an internal axis manifests itself as a velocity of zero at the pole, increasing with distance from the pole.

When $\ln(A/S)$ is plotted against Vt/R , a straight line is obtained, indicating the exponential dependence on Vt/R (Fig. 1). (Here, t is used rather than T for convenience. As they are proportional, the constant of proportionality, g , can be included in the axis scale, and still maintain a true representation of equation (1).) This dependence can be further verified by examining the limiting cases of velocity, thickness, and R . As $V \rightarrow 0$, or $t \rightarrow 0$, or $R \rightarrow \infty$, the lithosphere is effectively stripped away, and what remains is the sublithospheric area per upwelling, K . To be able to observe any of these limiting cases on the existing lithospheric plates would be an ideal test of equation (1). Clearly, $V \rightarrow 0$ would be the most likely possibility. On Fig. 1, this would correspond to the y -intercept, at $3.3 \times 10^6 \text{ km}^2$.

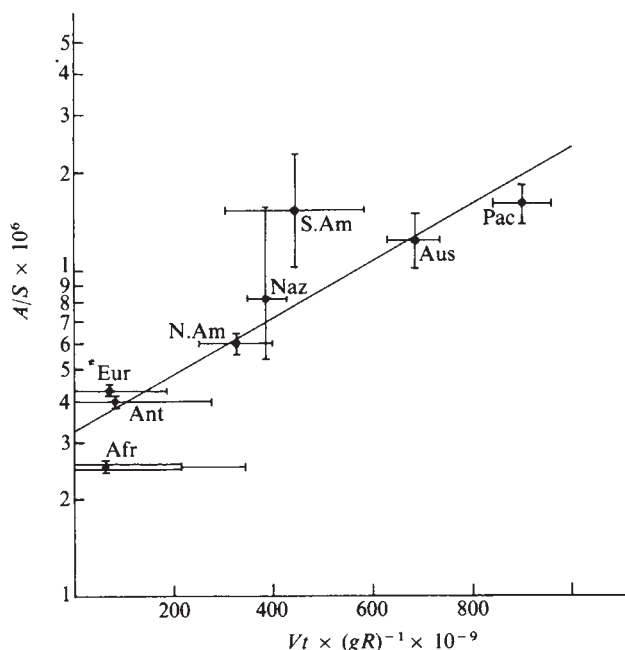


Fig. 1 Semi-logarithmic plot of (plate area/number of hot spots) versus (plate velocity $\times$ plate thickness). Horizontal error bars are minimum error associated with each plate from velocity and thickness errors. Vertical error bars represent ± 1 hot spot on each plate. The double bar for Africa indicates the true range of Vt , as its pole of rotation lies within the plate. The line was plotted using a least squares fit, yielding an intercept at $3.3 \times 10^6 \text{ km}^2$, with a correlation coefficient of 0.89, yielding a high degree of confidence (99.8%)^{29,30} that a correlation exists.

Table 1 Values of parameters in equation (1)

Plate	Hot spots	Plate area ($\times 10^{12} \text{ m}^2$)	Velocity ($\times 3.2 \times 10^{-10} \text{ m s}^{-1}$)	Average thickness (km)	Vt	A/S ($\times 10^{12} \text{ m}^2$)
Eurasia	16	68.0	0.5	156	78	4.25
Australia	5	61.0	6.0	115	690	12.2
Pacific	7	113.0	9.0	100	900	16.1
North America	10	58.8	2.5	136	340	5.9
South America	3	46.2	3.1	144	446	15.3
Nazca	2	16.4	6.0	65	390	8.2
Africa	31	78.4	0-1.5	144	0-216	2.5
Antarctica	15	59.9	0.6	133	80	4.0

Africa's rotation pole lies within the plate. This causes significant variations in the velocity within the plate. These variations are represented by the double line on Fig. 1. If Africa had a uniformly zero velocity, it would plot as a point on the y-axis, close to the intercept of the line described by all of the plates (except Arabian and Cocos).

The penetration ability of upwellings depends exponentially on Vt/R because as a magma loses heat to the lithosphere, its temperature falls exponentially with respect to time²⁶ from its initial temperature to the ambient temperature of the lithosphere. If the time necessary for the magma to penetrate the lithosphere is longer than it takes to cool and solidify, there will be no hot spot on the surface. There will, however, be volcanism if the magma reaches the surface more quickly. Plate movement reduces the time available for penetration, while incremental increase of the thickness and magma entry rate increases, and decreases the time necessary for penetration to the surface, respectively. The statistical number of upwellings that reach the surface is proportional to ΔT (see Fig. 2), which decreases exponentially as time necessary to penetrate the lithosphere is increased. Note that as upwellings under any plate are not likely to be all of the same strength (radius, temperature), but are distributed about an average, a plate with characteristics putting it at τ_c will allow half of its upwellings to reach the surface. Plates which lie further to the right will allow less than half, those to the left will allow more.

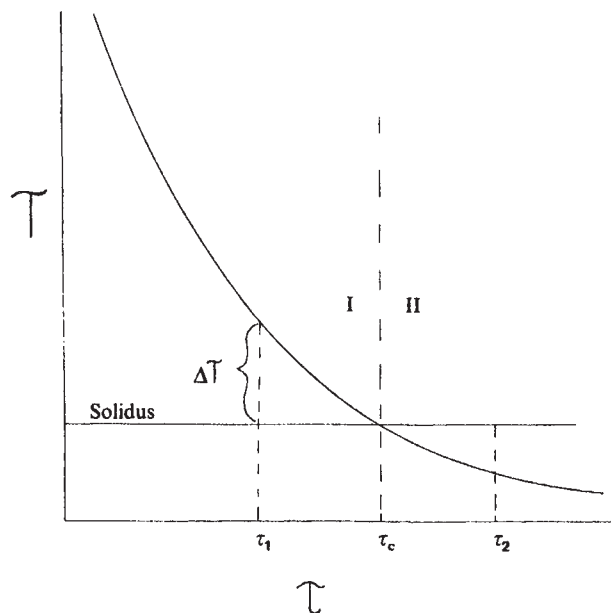


Fig. 2 Plot of temperature versus time for cooling magma. At points to the left of τ_c (region I), for example τ_1 , the plate is thin and/or slow enough to allow penetration to the surface of an average upwelling. In region II, for example τ_2 , average upwellings will not fully penetrate. At τ_c , half of the upwellings under the lithosphere will reach the surface. The statistical fraction of upwellings greater (I) or less (II) than 1/2 which reach the surface is proportional to ΔT .

The results show the sublithospheric area per upwelling to be $3.3 \times 10^6 \text{ km}^2$, or 1,800 km between adjacent upwellings. The uniformity of the sublithospheric upwelling density may have implications regarding the origin of hot spots. It would seem to indicate a periodic physical²⁷, rather than random chemical²⁸, driving mechanism, making a model such as a stratified fluid instability plausible²⁷.

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¹⁸O/¹⁶O variations in deep-sea carbonate shells from the Rise hydrothermal field

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Active hydrothermal vents have recently been discovered¹ on the East Pacific Rise near 21° N (Fig. 1). Exotic benthic communities similar to those at the Galapagos vents² thrive around many of the vents. Some of these animals have calcium carbonate shells which presumably contain a temperature record in the oxygen isotopes of the carbonate. These provide an opportunity to investigate the temperature preferences of the organisms and, perhaps, the history of temperature variation near the vents. We report here stable oxygen and carbon isotope measurements made on clam shells and on a barnacle collected near the RISE vents from the submersible Alvin at a water depth of ~2,600 m (Fig. 1).

The spreading centre here opens at a rate of 6 cm yr^{-1} , intermediate in the spectrum of spreading rates^{3,4}. The rise crest consists of an uplifted axial zone 5-km wide and 80-m high⁵. The zone of most recent volcanism is between 600 and 1,400 m wide⁶, and the hydrothermal vents occur within a strip only 300–500 m wide. They were mapped over a distance of 6.2 km along the spreading axis^{1,7}. Three types of vents were observed. The first kind have low temperatures ($10\text{--}20^\circ\text{C}$), with clear to slightly milky hydrothermal fluids oozing out at rates of a few cm s^{-1} (ref. 8). The second ('white smokers') have plumes of hydrothermal fluids clouded by white precipitates issuing from distinct chimneys at rates of tens of cm s^{-1} . Temperatures¹ are between 100 and greater than 300°C . The third ('black smokers') show the most vigorous activity ever observed at a sea floor hydrothermal vent. Waters blackened by sulphide precipitates jet out at rates of $1\text{--}5 \text{ m s}^{-1}$ and at temperatures of at least 350°C .

MacDonald and others⁹ estimate that the heat loss associated with a single black smoker chimney is 2–8 times the total theoretical heat loss for a 1-km segment of ridge out to 1 Myr age to either side. This underlines the importance of hydrothermal circulation in the heat budget of mid-ocean ridges, but also requires that vents of this type be very short-lived. There was, therefore, considerable interest as to whether the isotopic composition of the carbonate shells might provide an indicator of temperature history.

The principal complication in attempting to link shell growth history to vent history is that some animals live several tens of metres away from the highest temperature vents, and that the lateral thermal gradients are extremely high. Water temperatures only 5–10 m away from the vents may be within 5°C of ambient (2°C), because the rapid discharge of low density ($\rho \sim 0.62 \text{ g cm}^{-3}$)⁹ vent water draws in ambient water at a high rate.

We measured $^{18}\text{O}/^{16}\text{O}$ ratios on two shells from a new species of the vesicomyid clam, *Calymene*¹⁰, and on scales from a new genus and species of stalked barnacle, *Neolepas zeviniae*¹¹. Clam shell valve ALV 909B-1 was empty, while ALV 914B-1 was from a living specimen (see Fig. 2). The clam shells were recovered from near a 'cool vent' in which water temperatures between 5 and 20°C were measured. The barnacle was one of six removed from a rock (ALV 915) situated about 28 m from a 'black smoker'. Temperatures in this vent were not recorded because of instrument malfunction, but by analogy with similar vents the temperature of the actual water flow could reach 350°C .

Samples for isotopic analysis, each about 0.5 mg, were removed with a 0.5 mm drill from points along the growth direction of a valve from each clam at approximately every 2 mm, starting at the shell lip and extending a few centimetres back to include all of the prismatic layer still present in each shell. In the older parts of the shells, this absent layer had presumably dissolved. A few samples were drilled from the nacreous layer towards the hinge. We expected that such sampling of the original prismatic layer, across growth bands, would reveal any extensive temperature fluctuations experienced by the mollusc, at least during the last few years of its life. The samples from the barnacle consisted of single scales removed at $\sim 5 \text{ mm}$ intervals along the peduncle, beginning at the base of the capitulum. The clam shells were found to be aragonite by X-ray diffraction, and *N. zeviniae* hard parts are reportedly calcite¹¹.

All samples were heated at 250°C for 30 min in vacuum, before being isotopically analysed for $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ (isotopic ratios are expressed as δ values (PDB) by the usual procedure¹²). The analytical precision was 0.07%.

Figure 3 shows the results of the isotopic measurements on the three specimens. We first consider the $\delta^{18}\text{O}$ ranges observed. The $\delta^{18}\text{O}$ (PDB) values for clam A (909B1 empty) range from 3.2 to 4.3%; those for clam B (914B1 live) from 3.0 to 4.3%; and those for the barnacle from 3.8 to 4.4%. (Only the ranges of values measured on the clam prismatic layers are considered

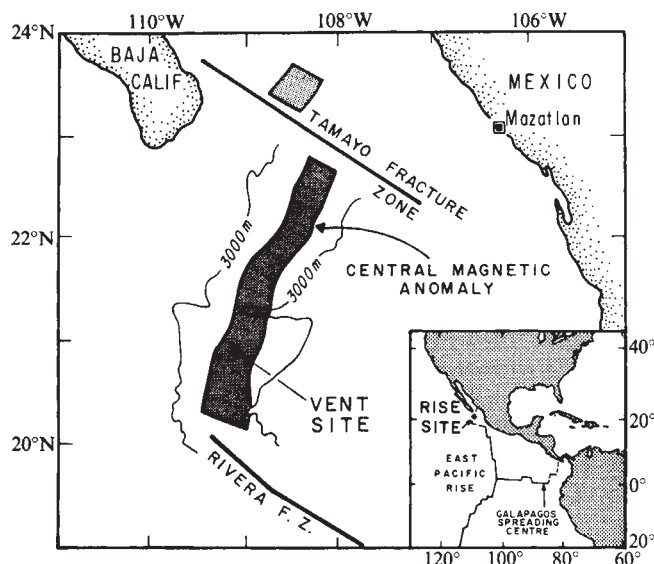


Fig. 1 Regional map.

here because of possible non-equilibrium precipitation of the nacreous layer.)

The measured $\delta^{18}\text{O}$ maxima from each shell are essentially the same, which suggests that they correspond to the temperature minimum (the ambient seawater temperature). Although a temperature corresponding to the maximum $\delta^{18}\text{O}$ value can be calculated, there are three sources of error: (1) there is disagreement of about 0.5° at near-zero temperatures between different palaeotemperature equations; (2) the difference between the isotopic fractionation behaviour of calcite (on which palaeotemperature equations are based) and that of aragonite is not known; and (3) the isotopic composition of the ambient seawater is not known exactly.

The empirical palaeotemperature equation has the form^{13,14}:

$$T(^{\circ}\text{C}) = A - B(\delta^{18}\text{O}_c - \delta^{18}\text{O}_w) + C(\delta^{18}\text{O}_c - \delta^{18}\text{O}_w)^2$$

where A , B , C are constants. The isotopic value of calcite is $\delta^{18}\text{O}_c$ and $\delta^{18}\text{O}_w$ is the isotopic composition of the water from which the calcite precipitated. We assumed a value for $\delta^{18}\text{O}_w$ of -0.3% for the water in which the clams grew¹⁵ (vent-water composition is not important in this context) and found for the maximum $\delta^{18}\text{O}_c$ value (4.3%) that the calculated temperature ($0^\circ\text{C} \pm 0.3$) was $\sim 2^\circ\text{C}$ lower than ambient. (Tarutani *et al.*¹⁶ experimentally determined the fractionation between inorganically precipitated calcite and aragonite as 0.6‰ at 25°C which, if applicable at low temperatures, would lead to a calculated minimum value of $1.8^\circ\text{C} \pm 0.4$, which is a more reasonable value.) In spite of uncertainties in the actual temperatures, a good estimate can be made of the temperature difference corresponding to the $\delta^{18}\text{O}$ range from 3.0 to 4.3%. This difference is $\sim 4.5^\circ\text{C}$, so that if the coldest temperature is 2°C , then the clams record a range from 2 to 6.5°C . It has been observed that the clams and some other animals found in similar vent regions of the Galapagos Rift occur some distance from the actual vent, apparently living in water close to ambient temperature¹⁷. From our data, it seems that the clams experience periods when the water is warmed by geothermal activity to at least 4°C above ambient temperature. This represents a minimum estimate, for we do not know the upper temperature limit at which the animal ceases to grow shell. A statistical study of shell rim material from a large number of clams might, however, provide clues to the upper tolerance limits for growth.

Five of the $\delta^{18}\text{O}$ values measured on the calcitic scales from the barnacle are in the range 4.2 ± 0.2 . Ignoring the palaeotemperature equation, this suggests that 4.2% corresponds to the ambient water temperature of 2°C as before, implying that the water/carbonate ^{18}O fractionation is similar for calcite and aragonite at these low temperatures. However, coincidental

agreement of maximum $\delta^{18}\text{O}$ values for the two taxa, resulting from different vital fractionation effects, cannot be ruled out.

Besides the ranges of the $\delta^{18}\text{O}$ values, the nature of the sequences is of interest. It seems that the progression of values in the clam shells shows coherence over a distance of some 10 mm, which presumably corresponds to a period of the order of one year (see below). There may be an indication of cyclicity in clam B, reminiscent of seasonal variations. It is perhaps not entirely unreasonable to expect some types of cyclicity in vent activity. Earth tides have shown to be important in triggering earthquake activity on Galápagos¹⁸ and elsewhere¹⁹⁻²¹. Thus one might expect that the strain produced by Earth tides could affect any diverted flow through cracks by changing the size and linkage of such cracks. Lunar temperature cycles could result from such a mechanism, and the growth lines observed in the clams (Fig. 2) might be profitably studied under this aspect.

We next consider the carbon isotope values. Expected $\delta^{13}\text{C}$ values for equilibrium precipitation at this site are between 1 and 1.5‰ (ref. 22) if we assume an ambient $\delta^{13}\text{C}$ of close to 0‰ (refs 23, 24). Observed ranges are -0.5 to -2‰ for the barnacle calcite, 3.5-5‰ for the prismatic layer of clam A and 1-2‰ for that of clam B. The nacreous 'repair' material in both clams is isotopically lighter than the prismatic layer.

Only the clam B record seems to show near-equilibrium values, although, in fact, equilibrium values for aragonite are not well established. Rubinson and Clayton²⁵ report a fractionation between aragonite and calcite at 25 °C of 1.8‰, which if similar at 2 °C, would suggest that the $\delta^{13}\text{C}$ value should be ~3‰ at equilibrium. Hence, the clam A record may be closer to equilibrium. In either case, the pronounced difference between the $\delta^{13}\text{C}$ value of clam A and clam B is puzzling. One might suspect

interference from organic matter in the analysis. However, both clams were treated in the same way, and it is not clear how a difference would be introduced through treatment.

If clam A values are near equilibrium, the lighter clam B values could be due to metabolic ^{12}C enrichment or vent-carbon effects, or both. Vent-carbon is expected to be depleted in ^{13}C compared to ambient bottom water, in analogy to volcanogenic carbon. The light values of the barnacle calcite must be considered in the same light.

To interpret the isotopic record in terms of history, we need a time scale. An estimated age of 6.5 yr from radionuclide ratios has been reported for a vesicomid clam from the Galápagos Rise hot spring field²⁶. Presumably, this estimate yields the correct scale for our clam samples. However, the fact that these clams are aragonitic may lead to erroneously young ages if substantial dissolution has occurred.

A valve of clam B (collected live) was cut in the growth direction to expose a cross-section (Fig. 2a). The shell structure from the lip (most recent growth) to approximately half way to the hinge consists of the sequential growth bands typical of molluscan prismatic growth. The remainder of the shell is a nacreous layer and is all that remains of the older portion of the shell where the prismatic layer has been dissolved away, due to the fact that the shells grow below the aragonite compensation depth for this region²⁷.

The mass of the nacreous growth is estimated to be roughly one-half of the mass of the remaining original growth. The nacre presumably also suffered attack and is likewise partly missing, being in turn made up for from below. Thus, a straight radiometric age determination on this specimen could be approximately half the true age. We have measured the average

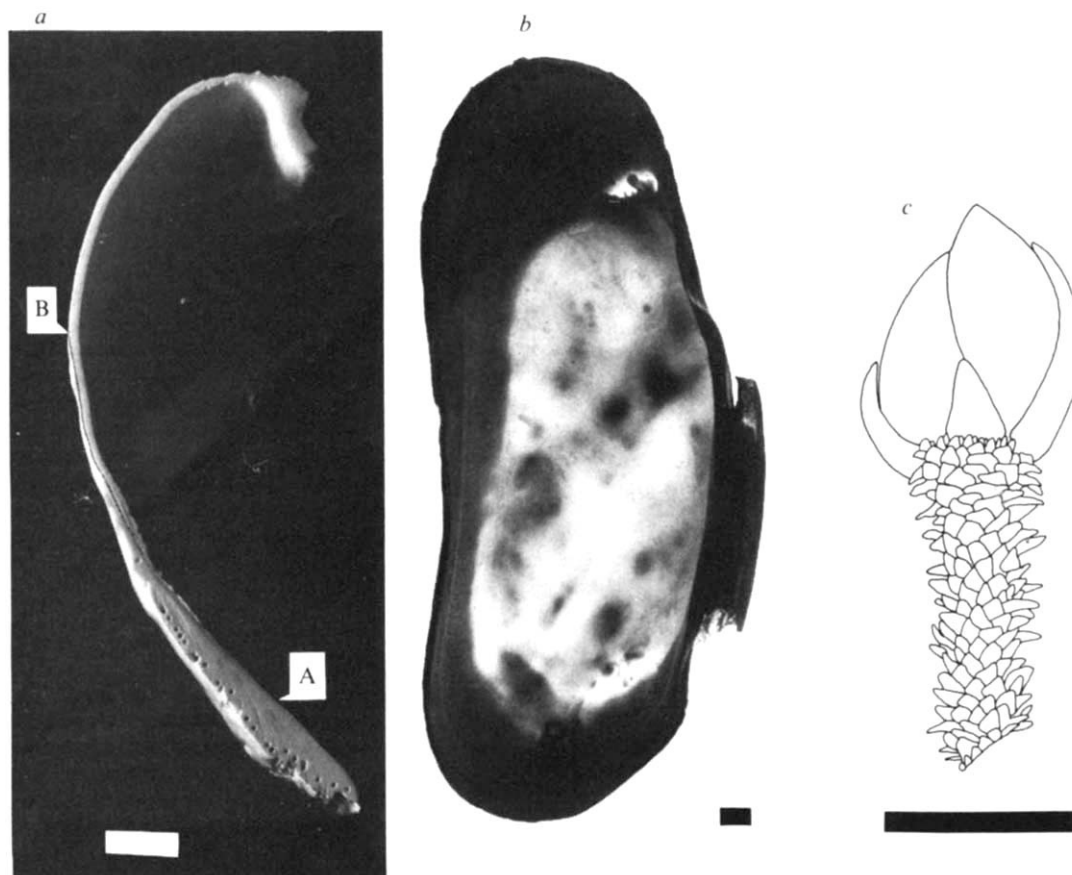


Fig. 2 a, Cross-section of clam shell (dorsal margin up) from a living *Calyptogena*, ALV 914 B-1 showing the decreasing thickness of the outer, prismatic, layer until none remains, dorso-laterally beginning at B. The inner secretion of nacre extends from A and increases in thickness until at B it constitutes the whole of the shell wall. Scale bar, 1 cm. (Some of the sample drill holes are apparent in the photograph.) b, Shell (dorsal margin to right) of a dead *Calyptogena*, ALV 909 B-1 illuminated with strong light from below. The light area represent secondary, nacreous, structure, while the opaque area indicates regions where all or part of the upper prismatic layer remains. Scale bar, 1 cm. c, Lateral view of the barnacle, *Neolepas zeviniae*, ALV 915-rock 4 (first paratype, British Museum (Natural History) reg. no. 1979-209). Note that the peduncle is clad with irregular whorls of scales. New whorls are added at the junction between the capitulum and the peduncle so that scales grade in age from the oldest at the point of attachment to the youngest immediately below the capitulum. Scale bar, 1 cm long.

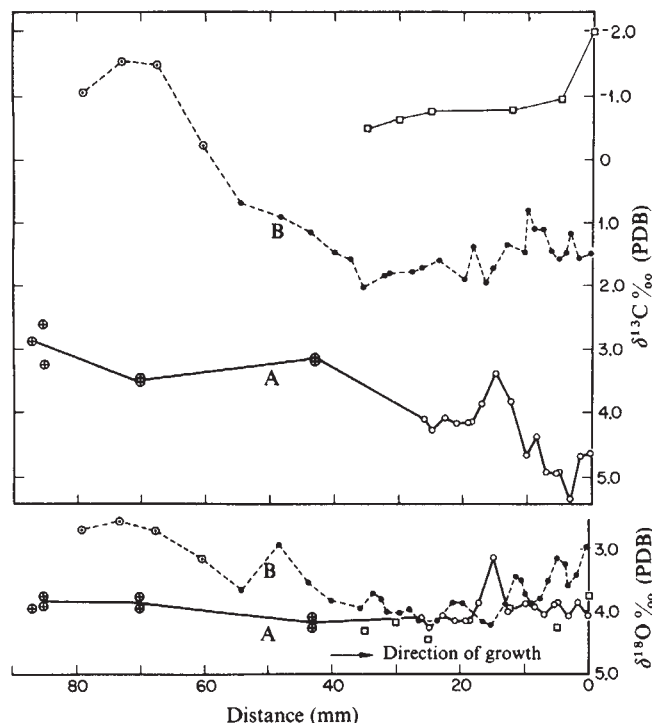


Fig. 3 $\delta^{18}\text{O}$ and $\delta^{13}\text{C}$ variations as a function of shell growth. Dead clam (A) *Calyptogena* (ALV909B-1); $\circ$, prismatic growth; $\square$, nacreous growth. $\square$, Barnacle *N. zeviniae* (distances for scales analysed are from capitulum to the point of attachment): holotype USNM cat. no. 172581. Living clam (B) (ALV914B-1): $\bullet$, prismatic growth; $\circ$, nacreous growth. Distances are from the margin of the shell (clam) and from the base of the capitulum (barnacle).

rate of dissolution (about $15 \text{ mg cm}^{-2} \text{ yr}^{-1}$) of clam fragments by suspending them at a depth of 2,700 m (lat. $0.0^\circ 0.2' \text{ N}$, long. $152^\circ 13.0' \text{ W}$) for 4 months. This rate of dissolution implies that an empty whole shell would dissolve in ~ 25 yr in the vent region.

Reactivation of a vent after dormancy might be indicated by clam assemblages in various states of preservation. Incidentally, the fact that vesicomyid clams have not been reported from the North Atlantic Ridge crest area, despite the excellent conditions for aragonite preservation there²⁷, may be in accord with the more slowly spreading rates, which would not be able to support the enormous heat loss associated with high-temperature, high-velocity RISE-type vents.

Our results show that the clams and the barnacle analysed grew at ambient to slightly higher-than ambient temperatures.

Hydrothermal leaks in the vicinity of the vents may influence the stable isotope record of the shells, as well as their growth, and organic carbon of chemosynthetic origin²⁸ may do likewise. Future work might focus on parallel examination of live-collected clams and other shelled organisms, to separate ambient from individual variations. The possibility of cyclicity in ambient conditions seems worth investigating; if cyclicity exists, it would provide instant age scales for carbonate-secreting organisms.

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Concentrations of ^{113}mCd in the marine environment

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Reports on the detection of ^{113}mCd in any type of environmental sample have been rare. The 113 mass chain yield is small relative to other longer-lived fission products, such as ^{90}Sr and ^{137}Cs , produced from uranium, plutonium and thorium fissions¹. Also, only a small fraction of the 113 chain yield decays to ^{113}mCd (ref. 2). Salter³ estimated that the $^{113}\text{mCd}/^{90}\text{Sr}$ activity quotient in thermonuclear fission should be 0.003. He stated that “this ratio is in good agreement with data from a few samples measured in the northern hemisphere prior to 1962 which have no ^{109}Cd ”. This, to our knowledge, was the first report of the detection of fission-produced ^{113}mCd in the environment. Salter³ also calculated that 0.062 MCi of ^{113}mCd and 0.25 MCi of ^{109}Cd were produced by activation during the atmospheric detonation of the 1.4-megaton Starfish device on 9 July 1962 over Johnston Atoll. As both ^{109}Cd and ^{113}mCd are produced during neutron activation of stable cadmium, and ^{109}Cd is not a fission product, the last part of Salter’s statement is significant. The absence of ^{109}Cd in samples collected before 1962 indicates that all nuclear testing before this time, which included all tests conducted at Enewetak and Bikini Atolls in the Marshall Islands, could have generated ^{113}mCd only as a fission product. It is therefore important to recognize that ^{113}mCd could be present in other environments contaminated with fission product wastes discharged to the aquatic environment from other nuclear facilities. ^{113}mCd has a half life⁴ of 14.6 ± 0.1 yr and decays predominantly by beta-particle emission. We present here a preliminary report of ^{113}mCd concentrations measured in sediment and tissue samples of marine organisms collected around different atolls in the Marshall Islands.

A series of pooled and individual fish liver and sediment samples were selected for analysis. It has been well documented that stable cadmium is concentrated by the liver of fish^{5,6}. Liver analysis offered the most sensitive test for presence of ^{113}mCd in the marine environment, especially at those atolls where only global fallout background concentrations could be anticipated in the environment. Gamma spectrometry was used to measure ^{113}mCd ($E_\gamma = 263.7 \pm 0.3$ keV; branching intensity 2.3×10^{-4})⁷ in large samples of ashed liver when activity levels were high, otherwise ^{113}mCd was measured on low-background beta counters following radiochemical separation of cadmium from other radioelements in the samples. The methods used to identify and detect ^{113}mCd in these samples are reported elsewhere⁸.

Concentrations of ^{113}mCd and other radionuclides detected in the samples are shown in Table 1. Some ^{113}mCd measurements were made during the 1972–73 radiological survey of Enewetak Atoll. As the relevance of these results has been overlooked,

Table 1 Radionuclide concentrations in fish liver samples from the Marshall Islands

Atoll	Scientific (common) name	Collection date†	⁶⁰ Co	^{113m} Cd	¹³⁷ Cs	pCi per g dry wt (%σ)* ¹⁵⁵ Eu	²⁰⁷ Bi	²³⁹⁺²⁴⁰ Pu	²⁴¹ Am
Bikini									
Island B-13	<i>Crenimugil crenilabis</i> (mullet)	10/78	20(1)	18.5(1)	NA	NA	NA	0.84(2)	NA
Island B-13	<i>Mulloidichthys samoensis</i> (goatfish)	10/78	14.2(4)	21.7(1)	ND	ND	1.1(9)	0.17(3)	NA
Island B-5	<i>Crenimugil crenilabis</i> (mullet)	10/78	33(1)	15.2(1)	0.72(12)	ND	ND	1.81(2)	NA
Lagoon	<i>Gymnosarda nuda</i> (tuna)	6/79	12.8(1)	105(29)	0.18(4)	ND	2.00(2)	NA	NA
Lagoon	<i>Carcharhinus menisorrh</i> (grey shark)	11/72	10.4(1)	413(1)	0.67(2)	0.13(2)	5.4(1)	0.147(2)	0.13(3)
Lagoon	<i>Trienodon obesus</i> (white tip shark)	11/72	24(1)	230(8)	0.12(19)	0.04(56)	2.5(4)	0.61(6)	0.36(12)
Lagoon	<i>Lutjanus bohar</i> (snapper)	10/78	32.2(2)	1,243(17)	0.066(27)	<0.2	0.67(17)	NA	NA
Enewetak									
Island E-7	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.30(1)	10.4(13)	0.026(3)	ND	0.11(2)	NA	NA
Island E-7	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.21(1)	12.9(9)	0.020(5)	ND	0.18(1)	NA	NA
Island E-7	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.27(1)	8.3(10)	0.016(4)	ND	0.24(1)	NA	NA
E-43	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.11(1)	3.5(26)	0.003(19)	ND	0.36(1)	NA	NA
E-43	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.31(3)	10.0(30)	0.020(8)	ND	1.6(8)	NA	NA
E-43	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.76(1)	24.4(20)	0.030(4)	ND	2.6(3)	NA	NA
E-1	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.14(1)	7.2(20)	0.024(3)	ND	0.47(1)	NA	0.012(30)
E-1	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.34(1)	12.2(19)	0.037(3)	0.006(46)	4.05(2)	NA	0.06(18)
E-44	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.57(1)	19.2(11)	0.024(9)	0.006(22)	1.28(2)	NA	0.037(18)
E-44	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	5.61(1)	28.2(7)	0.057(3)	0.017(23)	0.67(3)	NA	0.076(24)
E-44	<i>Carcharhinus menisorrh</i> (grey shark)	12/79	0.45(1)	4.5(32)	0.014(7)	ND	0.68(2)	NA	0.021(42)
E-9	<i>Trienodon obesus</i> (whitetip shark)	5.12(1)	95(20)	0.19(7)	0.09(27)	57(2)	NA	1.33(9)	
Rongelap									
Island F-13	<i>Crenimugil crenilabis</i> (mullet)	9/78	ND	30.0(2)	0.57(2)	ND	ND	1.56(4)	NA
F-13	<i>Scarus sordidus</i> (parrot fish)	9/78	ND	6.0(2)	ND	ND	ND	0.24(4)	NA
Bikar									
D-1	<i>Crenimugil crenilabis</i> (mullet)	9/78	0.54(14)	0.30(12)	<0.06	ND	ND	0.036(6)	NA
D-1	<i>Neomyxus chapitalii</i> (mullet)	9/78	ND	0.24(15)	ND	ND	ND	0.033(7)	NA
Ujelang									
J-22	<i>Crenimugil crenilabis</i> (mullet)	11/78	ND	0.21(40)	ND	ND	ND	0.012(23)	NA
Utirik									
I-1	<i>Mulloidichthys samoensis</i> (goatfish)	9/78	ND	0.19(14)	ND	ND	ND	0.003(26)	NA
Jemo									
S-1	<i>Acanthurus triostegus</i> (convict surgeon)	10/78	ND	0.41(11)	ND	ND	ND	0.143(3)	NA
Bravo Crater sediment		11/72	29(1)	2.7(1)	7.3(3)	44(3)	63(1)	78(2)	42(3)

NA, Not analysed. ND, Not detected.

* To convert pCi g⁻¹ to mBq g⁻¹, multiply values by 37. %σ, Per cent standard deviation of the counting error.

† Activity as of date of collection.

Table 2 Radionuclide concentrations in marine organisms from Enewetak Atoll

Scientific (common) name	Island	Sample	⁵⁵ Fe	pCi per g dry wt (%σ)* ⁶⁰ Co	⁹⁰ Sr	^{113m} Cd	¹³⁷ Cs	¹⁵⁵ Eu	²⁰⁷ Bi	²³⁹⁺²⁴⁰ Pu	²⁴¹ Am
Invertebrates											
<i>Tridacna gigas</i> (giant clam)	E-2	Kidney	108(4)	477(2)	0.52(40)	4.57(7)	<0.6	<2	19.7(3)	0.42(7)	0.41(5)
<i>Tridacna gigas</i> (giant clam)	E-8	Kidney	101(8)	1,570(1)	0.16(32)	100(2)	<1.3	<1	22.8(5)	0.21(5)	<0.2
<i>Tridacna gigas</i> (giant clam)	E-1	Kidney	87(1)	513(1)	1.09(16)	6.31(7)	<0.5	<0.5	17.5(3)	0.28(13)	0.23(7)
<i>Tridacna gigas</i> (giant clam)	E-2	Viscera	35(3)	108(1)	0.71(18)	1.16(17)	<0.2	0.9(30)	9.2(3)	7.43(1)	0.68(5)
Vertebrates											
<i>Chaetodon auriga</i> (butterfly fish)	E-9	Viscera†	1.67(1)	444(2)	0.98(7)	11.6(3)	<0.7	<0.4	<0.5	1.55(5)	0.23(6)
<i>Crenimugil crenilabis</i> (mullet)	E-9	Viscera	276(4)	104(1)	<0.02	3.07(2)	9.84(5)	25.3(2)	2.23(11)	24.2(2)	11.4(4)
<i>Acanthurus triostegus</i> (convict surgeon)	E-9	Viscera	201(7)	227(2)	17.7(6)	12.9(5)	6.12(7)	6.75(5)	1.10(20)	15.3(10)	3.56(5)
<i>Acanthurus triostegus</i> (convict surgeon)	E-2	Viscera	254(1)	17.8(2)	2.1(6)	13.0(2)	1.60(13)	0.67(21)	1.28(13)	2.23(12)	0.83(5)
<i>Scarus sordidus</i> (parrot fish)	E-2	Viscera	183(3)	151(8)	5.5(6)	5.90(6)	2.57(18)	1.42(50)	<0.3	2.89(3)	1.48(6)
<i>Euthynnus affinis</i> (bonito)	E-36	Light muscle	87(2)	1.3(13)	0.1	0.26(19)	0.68(13)	0.1	6.15(2)	<0.004	<0.3
<i>Euthynnus affinis</i> (bonito)	E-36	Dark muscle	572(1)	16.8(2)	0.1	0.50(14)	0.78(13)	<0.1	3.81(3)	<0.004	<0.1
<i>Euthynnus affinis</i> (bonito)	E-36	Liver	3,130(1)	20.9(2)	0.9	95.5(1)	2.8(8)	0.87(11)	1.66(10)	0.022(29)	<0.1
<i>Euthynnus affinis</i> (bonito)	E-31	Liver	649(1)	14.8(5)	0.4	32.3(1)	0.61(33)	<0.2	0.45(30)	<0.012	<0.009

* To convert pCi g⁻¹ to mBq g⁻¹, multiply value by 37. %σ, Per cent standard deviation of the counting error. Abstracted from data in ref. 8; activity as of 11/72.† Viscera sample includes all internal organs and gut contents which contain undigested sediment. Concentrations are biased by higher concentrations of transuranics, ⁵⁵Fe and others in sediment.

they are abstracted from the survey report⁹ and shown in Table 2.

^{113m}Cd was detected in all samples collected between 1972 and 1979 from Bikini and Enewetak Atolls and in fish liver samples from other atolls in the Marshall Islands, some of which were in the paths of close-in fallout debris from the nuclear tests conducted at Bikini and Enewetak. Radionuclides persist in these environments 21 yr after the last nuclear explosion at the atolls in 1958. ^{113m}Cd concentration was found to be lower in the surface sediment layer compared to ⁶⁰Co, ¹³⁷Cs, ²³⁹⁺²⁴⁰Pu, ²⁴¹Am and ²⁰⁷Bi, but it exceeds the concentrations of these radionuclides in the liver samples of Bikini tuna, shark and snapper by at least one order of magnitude. In the liver samples of Bikini reef species, ^{113m}Cd concentrations are at present comparable to ⁶⁰Co and greatly exceed the concentrations of the

other radionuclides we have measured so far. The concentrations of ^{113m}Cd in the liver samples of mullet, a herbivore, are much less than the concentrations detected in the pelagic carnivores at Bikini. This comparison suggests the concentration of ^{113m}Cd is greater in representative species from higher trophic levels. Fish from Bikini seem to contain more ^{113m}Cd in the liver than fish from Enewetak lagoon. Concentrations in the flesh of bonito from Enewetak Atoll are much lower than concentrations in the liver samples of the fish. This finding would have been predicted from the studies of stable cadmium distributions in fish. In 1979 grey reef sharks were taken from different quadrants of Enewetak lagoon (distinguished by the different island numbers in Table 1). Very different levels of radionuclides are associated with the lagoon sediments in each quadrant. There seems to be no significant relationship between

^{113m}Cd concentrations in the liver and the geographical region from which the sharks were caught. Although sharks in local regions of the lagoon may encounter very different levels of radioactivity, they appear to act more as 'averagers' as they probably travel into and out of the more or less radioactive parts of the lagoon continuously during their life.

Close-in fallout contamination is still evident in the marine environment at Rongelap Atoll, where concentrations of ^{113m}Cd in mullet liver samples are even higher than the levels detected in mullet collected from the eastern half of Bikini Atoll. The lowest concentration of ^{113m}Cd measured was ~ 0.2 pCi per dry weight in the liver of reef species from the atolls of Utirik, Ujelang and Bikar. ^{137}Cs activities in the flesh of these fish were in the range of fallout concentrations detected in the flesh of mullet samples collected in 1979 from North Carolina, USA (unpublished data). Therefore, the ^{113m}Cd concentrations in the atoll samples are probably representative of the levels expected at these latitudes from global fallout deposition.

It is not clear at this point if the current absence of reports of ^{113m}Cd concentrations indicates this radionuclide was measured but not detected, or that insufficient effort has so far been made to evaluate this radionuclide in environments receiving fission

product wastes. Although production of ^{113m}Cd during the fission event may be low, we have found that this radionuclide persists in the environment and is significantly concentrated in the liver of fish at the atolls. Concentrations presently exceed the levels of most other long-lived man-made radionuclides in liver by orders of magnitude. We have not yet generated sufficient data to evaluate its radiological significance in major dietary items at the Marshall Islands but these analyses are one of our main priorities. Stable cadmium is known to be concentrated by man in the kidney and liver⁷. As ^{113m}Cd will follow stable cadmium through the food chain—it decays by emitting energetic beta particles ($E_{\text{max}} = 0.58$ MeV)—and has a relatively long half life, the integrated radiological dose to man from ^{113m}Cd ingestion will be carefully assessed at the Marshall Islands and should also be assessed at other sites where contamination by this radionuclide may be suspected.

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The energetic cost of bipedal hopping in small mammals

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Recent studies of locomotion in red kangaroos (*Megaleia rufa*, 18–28 kg)¹ and Australian hopping mice (*Notomys cervinus*, 36.6 g)² have indicated that over a certain range of speeds the energetic cost of bipedal hopping locomotion may become independent of speed. As much as 70% of the decrement in kinetic energy which takes place when the kangaroo lands may be stored in elastic elements and used to propel the kangaroo upwards and forwards as it takes off (leaving only 30% to be added by the muscles)^{3,4}. Presumably, this same phenomenon could be exploited by all bipedal hopping mammals and it seems possible that at high speeds this specialized mode of locomotion could result in large energetic savings when compared with the costs incurred by a similarly sized quadruped running at the same speed. This hypothetical lower cost of locomotion has been invoked as an explanation for the rather common occurrence of bipedal hoppers among desert and plains dwelling mammals, as it might substantially reduce the energetic cost of foraging, or travelling, for animals utilizing widely dispersed resources^{5–7}. The purpose of the present study was to extend the assessment of the metabolic expense of bipedal hopping to a broader range of body sizes and mammalian taxa. Using indirect calorimetry we measured the cost of locomotion for three bipedal hopping eutherians (0.03–3.0 kg) and one bipedal hopping marsupial (1.1 kg). When properly trained for sustained running, none of the three species deviated from the expected quadrupedal pattern relating cost of locomotion to running speed, even while running fully bipedally. Bipedality must therefore be associated with benefits other than those related to the energetic costs of locomotion.

We trained two spring hares (*Pedetes capensis*, Rodentia; Pedetidae, average weight 3.0 kg), two rat kangaroos (*Bettongia penicillata*, Marsupialia; Macropodidae, average weight 1.1 kg), eight desert kangaroo rats (*Dipodomys deserti*, Rodentia; Heteromyidae, average weight 104 g) and one Merriam's kangaroo rat (*Dipodomys merriami*, 32 g) to hop on a treadmill while we measured their oxygen consumption. We considered the animals 'trained' when they would sustain one gait and position on the treadmill—5 min for the smaller *Dipodomys* and 20 min for the larger species—over a range of speeds. Naive and poorly trained animals would oscillate forwards and backwards along the treadmill at all but a few preferred speeds; these animals also frequently altered their gait when moving from the back to the front of the tread.

Rates of oxygen consumption were measured by enclosing the animal in a Plexiglas chamber which either slid along the tread (*Pedetes* and *Bettongia*) or enclosed it (*Dipodomys*). Air was metered through the box (1701 min^{-1} STP for *Pedetes* and *Bettongia* and 71 min^{-1} STP for *Dipodomys*). The difference in oxygen concentration (ΔO_2) between dry air entering and leaving the box was measured using an oxygen analyser (Applied Electrochemistry S-3A for the *Pedetes* and *Bettongia* and Taylor Servomex 0-272 for the *Dipodomys*). Rate of oxygen ($\dot{V}O_2$) consumption was calculated assuming a respiratory quotient of 0.8 by multiplying flow through the chamber by ΔO_2 and dividing by 0.9581 as described by Tucker⁸ for a similar system. The chambers were checked for leaks of exhaled air by decreasing the flow by 30% as the $\dot{V}O_2$ was monitored. This procedure should increase the magnitude of any loss of exhaled air: no difference was found between $\dot{V}O_2$ measured at either flow rate. In *Pedetes* and *Bettongia*, the rate of anaerobic glycolysis was determined by measuring the change in blood lactate concentration (using a Boehringer-Mannheim lactate test kit) over the timed interval of the run; comparison of resting to running levels of lactate production revealed no significant increase over the range of speeds for which $\dot{V}O_2$ was measured. Anaerobic glycolysis was not assayed in *Dipodomys* due to the adverse effect of blood sampling procedures on treadmill behaviour.

Rates of oxygen consumption increased linearly with speed in all four species of hopping mammals over a wide range of speeds which included the transition from completely quadrupedal to

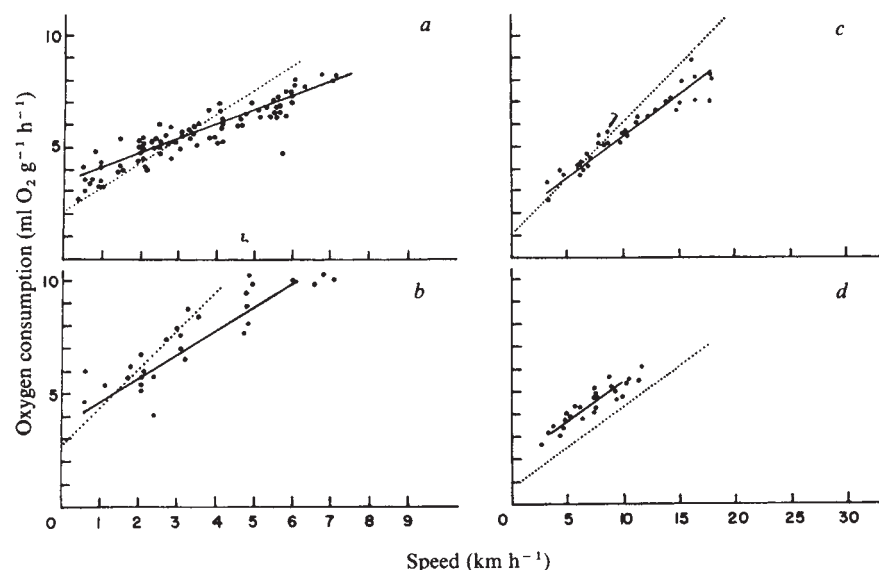


Fig. 1 Rate of oxygen consumption as a function of speed for four small species of bipedal hopping mammals. All values are for fully trained animals (moving at a constant gait and speed, that is, they used one gait and kept their position on the tread constant). Solid lines represent the least squares regression for the linear portion of each graph, where

$$\dot{V}O_2 \text{ (ml } O_2 \text{ per g h)} = \text{slope} \times \text{speed (km h}^{-1}\text{)} + y \text{ intercept}$$

- (a) *D. deserti*, 104 g, $N = 8$.
 $\dot{V}O_2 = 0.66 \times \text{speed} + 3.25$.
 $r^2 = 0.85$, $n = 95$, $P < 0.001$
 (b) *D. merriami*, 32 g, $N = 1$.
 $\dot{V}O_2 = 1.1 \times \text{speed} + 3.67$.
 $r^2 = 0.50$, $n = 25$, $P < 0.005$
 (c) *B. penicillata*, 1.1 kg, $N = 2$.
 $\dot{V}O_2 = 0.39 \times \text{speed} + 1.74$.
 $r^2 = 0.88$, $n = 33$, $P < 0.001$
 (d) *P. capensis*, 3.0 kg, $N = 2$.
 $\dot{V}O_2 = 0.34 \times \text{speed} + 2.04$.
 $r^2 = 0.67$, $n = 26$, $P < 0.001$

Dotted lines represent the predicted oxygen consumption for a running quadrupedal mammal of the same body size using the equation of Taylor, Schmidt-Nielsen and Raab¹⁵. Slopes of all four empirical regressions are not significantly different from those predicted (t -tests, $P < 0.05$).

completely bipedal locomotion (transition speeds were 3–5 km h⁻¹ for both *Pedetes* and *Bettongia*, 2–3 km h⁻¹ for *D. deserti* and 1.5–2.5 km h⁻¹ for *D. merriami*). These speeds also encompassed the normal foraging speeds of both *D. merriami* (average 3.2 km h⁻¹) and *D. deserti* (6.33 km h⁻¹)⁹; foraging speeds of *Pedetes* and *Bettongia* are unknown. This rate of increase was very similar to that predicted for a quadrupedal mammal of the same mass (dotted lines in Fig. 1). Although at higher speeds the predicted values are greater than those observed for hopping bipeds (Fig. 1a–c), those slight differences are not statistically significant and it seems unlikely that they would confer any biological advantage to bipeds. Plots of $\dot{V}O_2$ against speed for untrained animals gave a sigmoidal relationship similar to that reported by Dawson² for a 36.6-g hopping mouse. However, as training proceeded, our animals began to maintain a constant gait and eventually they would remain at the same tread location (near the front of the tread) throughout the run. In the latter conditions, the relationship between $\dot{V}O_2$ and speed was linear (Fig. 1), not sigmoidal. Apparently, the sigmoidal relationship between $\dot{V}O_2$ and speed was an artefact of the fore and aft motion of poorly trained animals; these animals were probably not in a steady state.

We conclude that hopping is not an energetically cheaper way to move for small hoppers (<3 kg) than quadrupedal locomotion. As the locomotory costs of small marsupial and rodent hoppers are similar to those of quadrupedal mammals of comparable body mass, we are left with two questions, one functional and one evolutionary. First, we have no immediate explanation for the differences in locomotory energetics of small (this study) and large (red kangaroos) hoppers; however, it is apparent that those differences do exist. Of the six species of small bipedal hoppers for which the energetic cost of locomotion has now been determined, only one, *Notomys cervinus*, shows any tendency towards an independence of cost and speed. Moreover, because a congener, *Notomys alexis*¹⁰, fails to show any evidence of an uncoupling of speed and energetic cost, it seems plausible that the unique pattern shown by *N. cervinus* may reflect training problems similar to those encountered in the present study. The red kangaroo data, on the other hand, do not seem to be methodological artefacts, and therefore the differences between large and small hoppers seem valid. Second, if there is no energetic advantage for small animals to hop, why do they do so? The majority of bipedal hopping mammals are

much smaller than *Megaleia*, and presumably would have locomotory costs qualitatively similar to the four species reported here. Thus, it would seem that the advantage of bipedal hopping locomotion does not, at least for most species, lie within the realm of locomotory energetics.

Our findings do not support models of small mammal foraging behaviour and resource partitioning based on the assumption that the locomotory energetics of small hopping bipeds differ appreciably from those of small quadrupeds. Small hopping bipeds display a wide variety of behavioural traits (for example, vertical leaping, rapid changes in direction) which suggest that the chief benefit of this mode of locomotion is related to enhancement of abilities to escape predation^{11,12}. These traits are accompanied by enlargement of both the visual and auditory apparatus; this further suggests that the morphology of small hopping bipeds has been moulded by their increased exposure to predation when foraging in the open areas of habitats with sparse vegetation^{13,14}. Thus, it seems most likely that the bipedal hopping mode of locomotion in small mammals has evolved as a result of its advantages for the avoidance of predatory attacks and not as an energetic strategy associated with foraging on dispersed resources.

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Direct evidence of sudden rise in fetal corticoids late in human gestation

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Glucocorticoids accelerate fetal lung maturation in all mammals studied¹, and in some species, such as goat and sheep, concentrations of fetal cortisol increase sharply before term, bringing about a train of events leading to parturition². Studies of cortisol in the umbilical cord blood have revealed no such increase at the end of human pregnancy. But information obtained in that way is difficult to interpret because much of the fetal cortisol is of maternal origin^{3,4} and its concentration, if sampled at delivery, is affected by maternal stress⁵. These problems can be avoided to some extent by studying other fetal corticoids. Corticosterone sulphate (once called compound B, and abbreviated to BS) is produced by fetal adrenal glands⁶ and is present in greater concentrations in human fetal plasma than in maternal plasma⁷. It is not hydrolysed by the placental sulphotases and is a poor substrate for placental 11 β -hydroxysteroid dehydrogenase⁸. We report here confirmation that the bulk of maternal BS originates from the fetus, and that its concentration increases suddenly at term.

We measured BS in maternal and fetal fluids of normal pregnant women, women carrying anencephalic fetuses and a pregnant woman whose adrenals had been removed 5 yr earlier because she had Cushing syndrome. Samples of plasma, urine and amniotic fluid were assayed for BS after solvolysis and chromatography as before⁹. Student's *t* test was used for statistical evaluations. As Fig. 1 shows, the mean ($\pm$ s.e.) concentration of BS in the plasma of non-pregnant women was 2.8 ± 0.3 ng ml⁻¹; at mid-pregnancy (21–32 weeks) it increased to 6.7 ± 0.5 ng ml⁻¹, and at term (38–40 weeks) it was about three times greater at 16.0 ± 3.2 ng ml⁻¹ ($P < 0.01$). A further increase was observed in women in labour (26.4 ± 6.3 ng ml⁻¹). During labour, those carrying anencephalic fetuses had a significantly lower plasma BS than normal (8.3 ± 0.4 ng ml⁻¹; $P < 0.02$). In the pregnant woman with no adrenals, the concentration of BS in the plasma, which was monitored throughout pregnancy, increased gradually during the second and third trimester, the most pronounced increase occurring in the last week before spontaneous labour began. Before delivery, her plasma BS was within the range seen at term in a normal pregnancy, and there was no further increase during labour.

Quantities of BS in 24-h urine collections of non-pregnant and pregnant women are shown in Fig. 2. The pattern of increase in urinary excretion of BS during pregnancy was similar to that in maternal blood. Serial measurements of BS in the urine of the pregnant woman with no adrenals increased sharply during the week preceding delivery. Excretion of BS by this patient at term reached the range seen in normal pregnancies. Figure 3 shows that in deliveries at mid-pregnancy there was only slightly less BS in umbilical cord blood than at term (54.5 ± 2.9 compared with 62.7 ± 6.7 ng ml⁻¹; $P > 0.1$). In the cord plasma of the infant of the woman with no adrenals, BS was normal (60 ng ml⁻¹). However, in anencephalic fetuses at term, the mean concentration of BS in cord blood (15.9 ± 3.0 ng ml⁻¹) was one quarter of that in normal infants at term ($P < 0.001$). In the woman without adrenals, the amniotic fluid, sampled at vaginal delivery, contained BS within the range of normal pregnancies (42.6 ng ml⁻¹), whereas in two women carrying anencephalic fetuses, BS was abnormally low in the amniotic fluid (3.9 and 6.2 ng ml⁻¹).

Plasma, BS
(ng ml⁻¹)

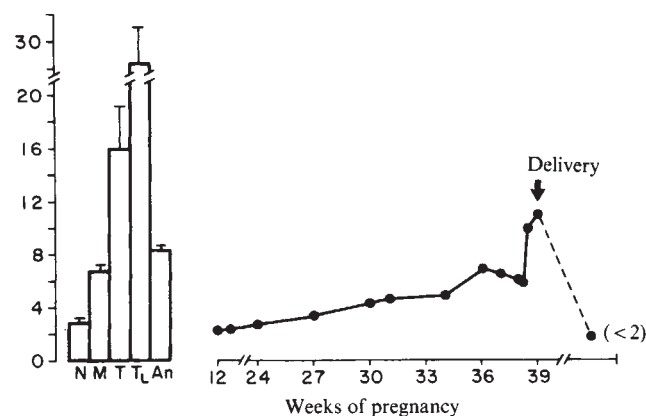


Fig. 1 Corticosterone sulphate (BS) concentrations in the maternal plasma of a woman with no adrenals at various stages of her pregnancy (●). Bars indicate concentration (mean $\pm$ s.e.) of BS in plasma of normal non-pregnant (N), and pregnant women at mid-pregnancy (M), term (T), and term in labour (T_L); and in women carrying anencephalic fetuses in labour at term (An). Replacement therapy with fludrocortisone (0.1 mg d⁻¹) and prednisone (7.5 mg d⁻¹) was given to the patient with no adrenals throughout pregnancy. BS was measured after solvolysis as corticosterone by radioimmunoassay (RIA), using an antibody prepared against cortisol-21-hemisuccinate-BSA; the antibody cross-reacted with cortisol (150%), cortisone (2%), deoxycorticosterone (17%), prednisone (12%), prednisolone (19%), and 17α -hydroxyprogesterone (24%). All these compounds were separated by chromatography before RIA⁹. There was less than 1% cross-reactivity with all other steroids tested. Blank values were always undetectable (<20 pg). The interassay coefficient of variation was less than 15%. Recoveries after extraction and chromatography were 77.7 ± 10.4 (s.d.). In calculating the concentration of BS, corrections were made for differences in molecular weight between conjugated and unconjugated steroids. No. of subjects: N, 8; M, 14; T, 8; T_L, 5; An, 7.

Urine, BS
(μ g d⁻¹)

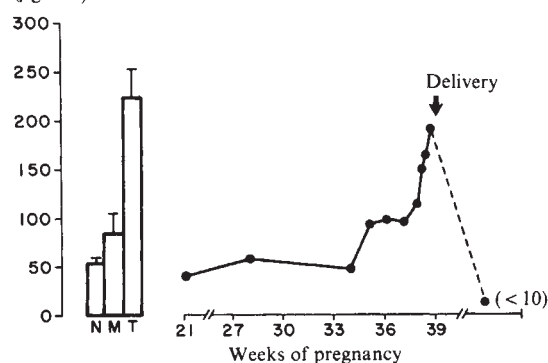


Fig. 2 Corticosterone sulphate (BS) concentrations in the urine of a woman with no adrenals at various stages of her pregnancy (●). Bars indicate values (mean $\pm$ s.e.) of BS in the urine of normal non-pregnant (N), and pregnant women at mid-pregnancy (M), and at term (T). No. of subjects: N, 6; M, 3; T, 6.

These data indicate that at least two thirds of BS in the maternal compartment at term is derived from the fetus: in women carrying anencephalic fetuses, concentrations of plasma BS at delivery were one third, and in umbilical cord plasma one quarter of that in normal pregnancies. Because the placenta cannot produce or metabolize BS, the increase in urinary and plasma BS throughout pregnancy indicates increased production by the fetal adrenal. This was confirmed because the woman with no adrenals had normal concentrations of urinary and plasma BS which increased during the last trimester, with the

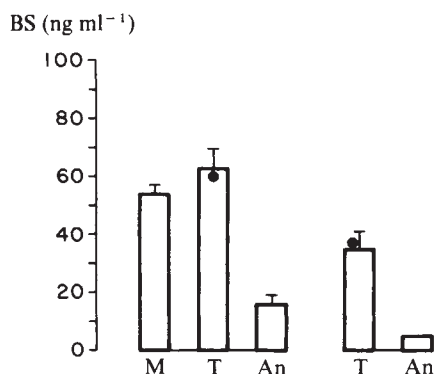


Fig. 3 Corticosterone sulphate (BS) concentrations in umbilical cord plasma and amniotic fluid of a pregnant woman with no adrenals (●). Bars indicate concentrations (mean $\pm$ s.e.) of BS in umbilical cord plasma (left) and amniotic fluid (right) of normal women at mid-pregnancy (M), and term (T), and in women carrying anencephalic fetuses in labour (An). No. of subjects umbilical cord: M, 7; T, 8; An, 6. No. for amniotic fluid: T, 8; An, 2.

most pronounced increase during the week preceding spontaneous labour.

The data obtained from the pregnant woman without adrenals, as well as the normal pregnant women, suggest that the production of BS by the fetal adrenals increases markedly near term. These results are compatible with the ultrastructural changes observed in the outer definitive zone of the fetal adrenal at term¹⁰, and the increase in the size of the human fetal adrenal towards term¹¹. Furthermore, conjugated cortisol also rises in amniotic fluid towards term¹², possibly a consequence of an increase in urinary excretion of this steroid by the fetus⁹. Thus, it is likely that the increased output of the fetal adrenal near term is not limited to BS but includes other corticosteroids. The fact that concentrations of BS in the umbilical cord plasma of normal infants at term were insignificantly higher than at mid-term, similar to results reported for other corticosteroids, does not exclude an increase in fetal adrenal production near term, for the rate of fetal metabolic clearance of BS into the maternal circulation is likely to be greater in late than in mid-pregnancy.

In humans, corticosteroids have not been implicated in the mechanisms leading to labour; however, they may well have a role in initiating other physiological changes, among them fetal lung maturation. In some species, such as the rabbit, the time of augmented surfactant production is preceded by an increase in fetal circulating unbound cortisol¹³, together with an enhancement in the capacity of the lung to convert cortisone to cortisol¹⁴. Unconjugated corticosterone is a potent glucocorticoid that binds to specific lung receptors in the human¹, and in the rabbit it induces production of lung surfactant¹⁵. However, it is not known whether corticosterone in its conjugated form binds directly to lung receptors, or whether it can be hydrolysed by lung sulphatases, thus becoming biologically active. Further studies are necessary to clarify whether there is a direct association between increased fetal adrenal corticoid output, as determined by maternal concentrations of BS, and the status of fetal lung maturation, determined by analysis of concentrations of surfactant in the amniotic fluid.

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Expulsion of *Nippostrongylus brasiliensis* by mice deficient in mast cells

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Expulsion of the intestinal helminth, *Nippostrongylus brasiliensis*, occurs spontaneously about 2 weeks after a primary infection of rats¹ and mice². Cellular changes in the small intestine coincident with the period of expulsion have suggested several mechanisms by which this 'self-cure' may be effected. Local anaphylaxis was proposed as a possible means of parasite clearance; this hypothesis has been supported by the demonstration of specific reaginic antibody production³ and jejunal mast cell accumulation⁴ in infected animals. In addition, increased mucus secretion⁵ and more recently, goblet cell proliferation in the jejunal mucosa of rats⁶ have been noted and considered as potentially important in mediating the self-cure reaction. The data presented below indicate that in the absence of demonstrable mast cells, the course of a primary infection with this parasite is unchanged; however, they are supportive of a role for goblet cells in the self-cure reaction.

We have taken advantage of the availability of mast cell-deficient mice to examine the relative contribution of mast cells and goblet cells to the expulsion of *N. brasiliensis*. Either of two mutant alleles at the dominant-spotting locus of the mouse genome, *W* or *W^v*, when homozygous, is associated with a defect in a pluripotential stem cell precursor⁷. The hybrid *W/W^v* mouse, resulting from a cross between *WB/Re-W/+* and *C57Bl/6-W^v/+* parents, is grossly deficient of tissue mast cells, as documented by Kitamura *et al.*⁷. The immunological status of these mice with respect to thymus-derived and bone marrow-derived lymphocytes, however, seems to be normal⁸. *W/W^v* mice, their sex-matched littermates without the mutant alleles (*+/+* mice) and *C57Bl/6* mice (a background strain), were used in this study.

Kitamura *et al.*⁷ demonstrated the mast-cell deficiency of *W/W^v* mice in histological studies of toluidine blue-stained sections of numerous tissues, including skin and gastrointestinal tract. We confirmed their findings, and obtained the following additional functional and biochemical evidence. First, we found no mast cells in the jejunal mucosa of *W/W^v* mice. Mast cells were quantitated histologically by removing the entire small intestine and fixing immediately in Carnoy's solution. Sections 2 cm in length were taken from the jejunum and dehydrated in an ascending ethanol series and cleared in α -terpineol. They were then embedded in paraffin and 8- μ m sections were cut with a standard microtome. Slides were prepared and stained with astrablau-safranin to demonstrate mast cells⁹. In examining a total of 800 villus-crypt units (VCU) from four *W/W^v* mice, no mast cells were found. In contrast, there was an average of 0.7 ± 0.13 mast cells per 10 VCU in each of four *+/+* controls. This number was somewhat less than that reported in mice of other strains; for example, Swiss-Webster mice may have 25 mast cells/10 VCU (ref. 10). Next, the absence of a passive cutaneous anaphylactic reaction in five 8-week-old male *W/W^v* mice, tested in the same way as three *+/+* controls who were consistently reactive recipients in this assay, provided functional evidence for the mast cell deficiency in their skin. Finally, peritoneal cells from *W/W^v* mice were shown to lack mast cells, as well as histamine, a biochemical marker for mast cells. Thus, a 20- μ l aliquot of an aspirated peritoneal cell suspension from each of five *W/W^v* mice was consistently negative for histamine

using a radioenzymatic technique¹¹, while a similar aliquot taken from each of three $+/+$ controls contained an average of 3.6 ± 0.8 ng histamine. (The lower limit of detection of histamine by this method is 0.1 ng per 20 μ l.) Examination of samples of the aspirated cells, stained according to Mota's technique¹², confirmed that equal numbers of cells were present in all of the suspensions assayed, although mast cells comprised $11.2 \pm 3.6\%$ of the cells from $+/+$ mice whereas no mast cells were detected among the cells obtained from W/W^v mice. Observations of the profound mast cell deficiency in the W/W^v mouse are thus supported and extended.

In normal mice, infection with *N. brasiliensis* is associated with a marked (at least tenfold) increase in mast cells in the jejunal mucosa¹⁰. Not surprisingly, this was not the case in W/W^v mice. After infection by the subcutaneous injection of 500 third-stage larvae of *N. brasiliensis*, no mast cells appeared in the jejunum of W/W^v mice up to day 21 after infection, whereas mast cell numbers did increase in $+/+$ mice (Fig. 1). Of 24 W/W^v and 24 $+/+$ mice, equal numbers of randomly selected animals were killed immediately before and on days 7, 10, 14, 17 and 21 post-infection, and their intestines prepared as described above. It was not until after day 7 post-infection that the number of mast cells began to increase in mice of the $+/+$ genotype. Mast cell accumulation continued up to day 21 in this control group.

The course of worm infection, however, determined by killing mice and counting the number of adult worms in the small intestine, was found to be parallel in W/W^v and control mice (Table 1). The course in all three groups of mice was similar to that reported with C3H mice². On day 7, adult worms were found predominantly in the first third of the intestine (measured from the pyloric sphincter) in each group of mice. On day 14, no worms were found in any mouse except one, a C57Bl/6 mouse, which had retained a single worm.

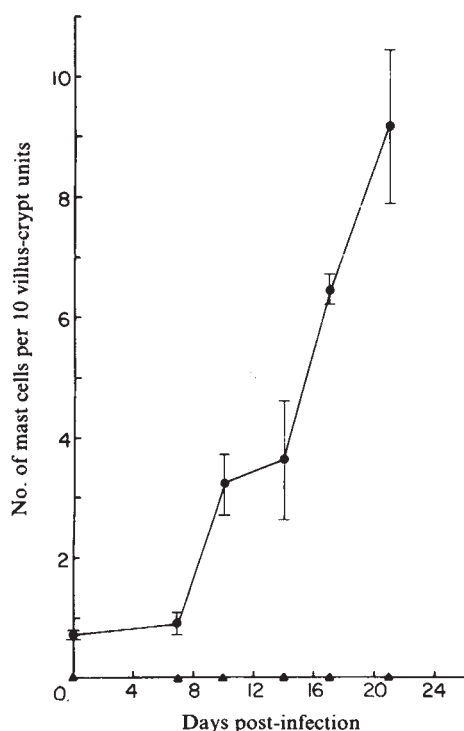


Fig. 1 Intestinal mast cell counts of W/W^v (▲) and $+/+$ (●) mice during the course of primary infection with *N. brasiliensis*. Twenty-four mice of each genotype were infected on day 0, each with 500 infective third-stage larvae; four of each group were sacrificed on each of the indicated days. A minimum of two sections from each mouse was examined at 400 $\times$ magnification to identify and count mast cells in the villus-crypt units (VCU). At least 40 VCU in each tissue sample were examined; in sections from W/W^v mice, at least 100 VCU were examined. Standard errors are indicated by vertical bars.

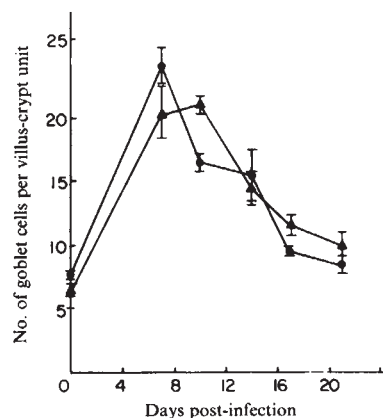


Fig. 2 Intestinal goblet cell counts of W/W^v (▲) and $+/+$ (●) mice during the course of a primary infection with *N. brasiliensis*. Sections contiguous with those stained for mast cells (refer to Fig. 1) were stained with alcian blue-periodic acid Schiff's⁶ and examined for goblet cells (400 $\times$). At least 40 VCU in each tissue sample were examined. Standard errors are indicated by vertical bars.

At the same time, increases in goblet cell numbers were apparent in both groups of infected mice, as seen in Fig. 2. Before infection, W/W^v mice appeared to have fewer goblet cells than $+/+$ mice. On day 7 post-infection, there was a 2–4-fold increase in the number of goblet cells in the jejunal epithelium in both groups of mice. By day 10, the number of goblet cells had already begun to decline, approaching the pre-infection level by day 21. These observations are supported by the appearance of increased mucus content in the jejunum in the regions containing the most worms, particularly evident on day 7. It is noteworthy that intestinal goblet cell numbers and mucus secretion during the course of *N. brasiliensis* infection in these animals more closely paralleled the intestinal worm burden than did the number of intestinal mast cells. These observations are comparable to those made in rats infected with the same parasite^{4,6}.

Mast cell infiltration of the intestinal mucosa in the presence of specific IgE antibodies has long been taken to suggest that a local anaphylactic-like mechanism is involved in the expulsion of *N. brasiliensis* from the rodent intestine. Nevertheless, involvement of these cells has been questioned¹³. As demonstrated in the present experiment, expulsion in the absence of intestinal mast cells is strong evidence that mast cells and an anaphylactic-like reaction are not necessary for the effective clearance of intestinal worms after a primary infection. Furthermore, the maximum accumulation of intestinal mast cells in normal mice and rats occurs post-infection, which suggests that these cells may have some other role in this process. For example, they may serve a regulatory function¹⁴.

The hypothesis that intestinal goblet cells play a part in the host response to helminthic parasites, suggested as early as

Table 1 Worm burden found in the small intestines of mice of different genotypes on days 7 and 14 post-infection

Genotype	Mean worm burden	
	Day 7	Day 14
WBB6 F ₁ - W/W^v	132 $\pm$ 19*	0 $\pm$ 0.0
WBB6 F ₁ - $+/+$	128 $\pm$ 22	0 $\pm$ 0.0
C57Bl/6	105 $\pm$ 11	0 $\pm$ 0.25

Groups of eight W/W^v , eight $+/+$ and eight C57Bl/6 mice were infected by the injection of 500 third-stage larvae. Four per group were killed on the indicated days; the small intestine was removed and placed in saline solution. Under direct microscopic observation (10 $\times$), the intestine was opened longitudinally in 1 cm segments and the adult worms identified and counted. Values are means $\pm$ s.e.m.

* Differences on day 7 are not statistically significant ($P > 0.70$).

1939¹⁵, was later invoked in *N. brasiliensis* infections⁵ and is reinforced by the current data. The apparent increase in the secretion of mucus, much of which might be due to the increased numbers of goblet cells, could provide a means for the mechanical expulsion of the worms as well as being a vehicle for the delivery of host antibody and cells to the site of parasite attachment. The roles of goblet cells, mast cells and their products in immunity to helminths is deserving of further investigation.

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Haematopoietic growth factors are released in cultures of H-2-restricted helper T cells, accessory cells and specific antigen

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Colony formation by mammalian haematopoietic cells in culture depends on specific glycoprotein growth factors. 'Colony-stimulating factors' for granulocytic^{1,2} and macrophage^{3,4} precursors (G- and M-CSFs), as well as for pluripotential and early committed erythroid cells ('burst promoting activity, or BPA') are released in cultures of stimulated spleen, lymph node or peripheral blood cells^{5–8}. In such systems the induction stimulus has been either allogeneic cell antigens, or lectins which stimulate T cells, such as pokeweed mitogen, concanavalin A (Con A) or phytohaemagglutinin⁶ and both T cells and adherent cells are implicated in the process^{7,8}. The complexity of the spleen cell populations used in earlier *in vitro* studies has made it difficult to establish the cellular source of the factors and the mechanisms leading to their release. Pure populations of continuously growing cell lines of either monocytic/macrophage¹ or T-cell^{10,11} character have already been shown to release haematopoietic activities constitutively. However, such evidence cannot prove that these functions are also expressed by the normal counterparts of these lines. We have reduced the complexity of the spleen cell system by supplying helper T cells in the form of pure clonal populations of known physiological function and antigenic specificity, and demonstrate here that activity is released when helper T cells are cultured with specific antigen, and that release depends on H-2 (I-A region) restricted interaction with accessory cells present in spleen or normal peritoneal cavity.

Table 1 indicates that activities stimulating granulocytic, macrophage and large erythroid colonies are released during secondary immune responses by mouse spleen cells in culture. In this experiment, C57BL/6J mice were immunized intravenously with 10⁷ sheep or horse red blood cells. After 3

weeks, spleen cells were cultured in serum-free medium with or without added antigen. Immunological reactivity was monitored by enumerating antibody-producing (plaque-forming) cells (PFC) specific for the challenging antigens after 5 days of culture. The supernatant culture medium was collected after 5 days and assayed for growth-promoting activity in methyl-cellulose cultures of mouse bone marrow. In these culture conditions, unstimulated spleen cells spontaneously released a background level of activity. However, factor release rose above this background when the cultures contained antigen, correlating positively with the immune responses indicated by PFC. Factor release was most marked when the cells were challenged in culture with the same antigen to which they had been immunized *in vivo*. As also indicated in Table 1, when the spleen cells were treated with an anti-Thy-1 antiserum plus complement before incubation with antigen, no induction of activity above background occurred.

Such experiments documented that recognition of antigen by normal T cells could result in release of haematopoietic factors. However, interpretation of data from this system is complicated by the complexity of the spleen cell population, and the high background levels of factor release even in the absence of homologous antigen. For this reason, we turned to a system in which the functional T-cell component was represented by pure cloned antigen-specific helper T cells. Such cells were derived from immunized spleen cells selectively enriched in culture by repeated antigenic stimulation and finally cloned out in the presence of T-cell growth factor (IL-2)^{12–14}. As seen in Table 2, significant haematopoietic factor activity was released in cultures of T-helper-cell clones, but only when both appropriate antigen and syngeneic 'accessory cells' were also present. The latter were provided either by spleen cells (10⁶ ml⁻¹) from nude mice devoid of functioning T-helper cells, or more efficiently on a per cell basis by resident peritoneal cells (5 × 10⁴ ml⁻¹).

Function of helper T cells in immune responses is known to involve cellular interactions. These include recognition of major histocompatibility antigens on B cells and 'accessory' cells¹⁵ by the T cells. In the mouse, these antigens are the I-A antigens of the H-2 complex. We accordingly investigated whether the induction of haematopoietic factors in this system would also be H-2 'restricted'. Pure cloned antigen-specific helper T cells were cultured with appropriate antigen, and with accessory cells from congenic mouse strains recombinant at various points in the H-2 region. The accessory cells were supplied in the form of irradiated spleen cells from which mature T cells had been eliminated by treatment with anti-Thy-1 antiserum and complement. The results, also shown in Table 2, indicate that induction of factor release depends on identity of genotype between the T-helper cell donor and the accessory cells to the left of the I-B subregion of H-2. In a control experiment of the type shown in Table 1, the negative accessory cell donors (B10A.4R and B10A) were comparable to C57BL/6J in their capacity to release CSFs and BPA in response to antigen. Appropriate congenic strains were not available at the time of these experiments for distinguishing between K- or I-A-region restriction. However, the action of T-helper cells in immune responses *in vivo* has been demonstrated to involve restriction only at I-A¹⁵.

Our experiments indicate that haematopoietic factors are released during immune responses to red cell antigens in culture. The experiment in Table 1 documented such release with normal freshly explanted spleen cell populations. Experiments using pure clonal helper T-cell populations reproduced this phenomenon, providing an additional point of resemblance in function between these clones and their normal counterparts in spleen. In addition, the experiments showed that such release requires both recognition of the antigen by antigen-specific helper T cells, and an interaction, restricted most probably at I-A, between these T cells and Thy-1-negative accessory cells present in spleen or peritoneal cavity. Factors stimulating T (ref. 14) and B (ref. 16) cell growth have already been shown to be released in the same system with similar requirements.

Table 1 Induction of haematopoietic factor release in spleen cell cultures during a specific antibody response*

Antigen exposure		Treatment before culture	PFC† against:		Colonies induced by supernatant‡ (% of control)		
<i>In vivo</i>	<i>In vitro</i>		SRC	HRC	MΦ	G	E
No conditioned medium control stimulant§					5±2 100±7	0 100±28	3±2 100±13
—	—	—	0	0	55±5	48±16	18±5
—	SRC	—	2,020	0	67±5	48±16	30±6
—	HRC	—	0	965	71±5	48±16	42±7
SRC	—	—	16	0	70±5	43±15	31±6
SRC	SRC	—	68,800	20	100±6	96±23	102±1
SRC	HRC	—	400	1,390	91±6	68±19	62±9
HRC	—	—	72	8	65±5	21±11	26±6
HRC	SRC	—	2,820	10	72±5	48±16	23±5
HRC	HRC	—	70	79,900	92±6	80±21	74±10
SRC	SRC	α-Thy-1 + C'‖	5,520	0	35±4	5±5	7±3
SRC	SRC	C'	52,200	0	100±6	80±21	91±11
HRC	HRC	α-Thy-1 + C'	120	6,030	84±6	7±7	21±6
HRC	HRC	C'	128	71,400	130±7	120±25	98±11

* 1 ml cultures contained 2.5×10^6 C57BL/6J spleen cells and 5×10^6 red cells where indicated, in serum-free IMDM containing albumin, transferrin and soybean lipids^{17,18,20}. They were incubated in 10% CO₂, 8% O₂ and 82% N₂ on a rocking platform¹⁸. SRC, Sheep red cells; HRC, horse red cells.

† Direct plaque-forming cells per culture against specific red cell antigen¹⁹.

‡ 1 ml methylcellulose cultures contained 1×10^5 C57BL/6J bone marrow cells, 4% fetal bovine serum, albumin, transferrin and soybean lipid 20, 2 units of human urinary erythropoietin 20, and 0.08 ml of supernatant from the immune response cultures. Colonies were counted after 9 d of culture. Results are pooled from three independent assays. Colony numbers are expressed as per cent of control ± the s.e.m. expected of Poisson-distributed counts. MΦ, macrophage and mixed neutrophil/macrophage colonies; G, large disperse granulocytic colonies of the type identified by Metcalf and co-workers as eosinophilic^{21,22}; E, erythroid or mixed myeloid/erythroid bursts. Mixed myeloid/erythroid bursts comprised about one-third of the erythroid colonies.

§ Conditioned medium from pokeweed mitogen stimulated mouse spleen cells; partially purified by adherence to Con A-Sepharose followed by chromatography on Sephadex G-150 (ref. 5). Absolute counts were: MΦ, 68; G, 5; E, 21 per 10^5 marrow cells.

‖ Incubation with AKR anti-Thy-1.2 antiserum + rabbit serum complement.

Table 2 Antigen-specific and H-2-restricted induction of factor release in cultures of helper T cells and accessory cells

T-cell specificity	Source	Accessory cells			Antigen	Colonies induced by supernatant (% of control)		
		K	H-2 haplotype	D		MΦ	G	E
			I ABJEC					
No conditioned medium control stimulant						0 27±3	0 17±10	0 70±11
HRC	nu/nu spleen*	b	bbbbb	b	HRC	100±5	100±20	100±10
—	nu/nu spleen	b	bbbbb	b	—	0	0	3±2
HRC	—	—	—	—	HRC	0	0	2±2
HRC	nu/nu spleen	b	bbbbb	b	—	15±2	15±8	18±4
HRC	nu/nu spleen	b	bbbbb	b	SRC	6±1	8±5	23±5
HRC	Peritoneal cells†	b	bbbbb	b	HRC	76±4	96±19	95±10
SRC	nu/nu spleen	b	bbbbb	b	SRC	78±4	85±18	91±10
SRC	—	—	—	—	SRC	0	0	18±5
SRC	nu/nu spleen	b	bbbbb	b	—	22±2	4±4	23±5
SRC	nu/nu spleen	b	bbbbb	b	HRC	22±2	12±7	15±4
HRC	C57BL/6J spleen‡	b	bbbbb	b	HRC	72±4	142±23	51±7
HRC	B10.A(4R) spleen‡	k	kbbbb	b	HRC	1±1	0	1±1
HRC	B10.A(5R) spleen‡	b	bbkkd	d	HRC	62±4	131±22	85±10
HRC	B10.A spleen‡	k	kkkkd	d	HRC	0	0	4±2
SRC	C57BL/6J spleen‡	b	bbbbb	b	SRC	39±3	65±16	47±7
SRC	B10.A(4R) spleen‡	k	kbbbb	b	SRC	6±1	12±7	11±3
SRC	B10.A(5R) spleen‡	b	bbkkd	d	SRC	64±4	85±18	64±8
SRC	B10.A spleen‡	k	kkkkd	d	SRC	14±2	23±9	10±3

Cultures (0.2 ml) contained 3×10^3 helper T cells (C57BL/6J), accessory cells and 5×10^5 red cells where indicated. Serum-free medium and incubation as in legend to Table 1, except that cultures were not rocked. Supernatant medium was collected after 5 d of culture and assayed in methylcellulose cultures of 7×10^4 bone marrow cells ml⁻¹ as in Table 1. Colony counts, pooled from two independent assays, are expressed as per cent of the counts (MΦ, 200; G, 12; E, 45 per 10^5 marrow cells) in the first experimental group (HRC, nu/nu spleen, HRC).

* 2×10^5 per culture.

† 1×10^4 per culture.

‡ Congenic spleen cells were irradiated (3,000 rads) and treated with α-Thy-1 serum plus complement before addition (2×10^5 cells) to the cultures.

While it is easy to understand the usefulness of production of lymphopoietic factors under these circumstances, and perhaps of activities stimulating monocyte/macrophage and granulocyte production, the significance of BPA production is less obvious. Perhaps it serves to increase the supply of early committed myeloid and lymphoid precursors⁵. There is an indication that T cells may play a role in stimulating haemopoietic cell growth in the whole animal: Thy-1-bearing cells appear to be required for successful growth of haemopoietic cell grafts in genetically defective *W/W^v* mice⁹. Since the T-cell deficient nude mouse does not show any gross defect in haematopoiesis, we would suggest that antigen recognition is unlikely to represent the only available pathway to haematopoietic factor production. Perhaps both immunological and non-immunological signals impinge on a cellular system present in the accessory cell population and able to release factors in response to either signal. The presence of a functioning accessory cell population in *nu/nu* spleen is

documented in Table 2. The view would predict that haematopoiesis in *nu/nu* mice will prove defective in relation to their normal counterparts only when comparisons are performed during immune reactions.

The experimental system described here, which makes use of pure cloned helper T cells in combination with T-cell-depleted accessory cell populations, provides a basis for specific assay both of the accessory cells and of the signals exchanged between them and helper T cells. Because the culture system contained a source of accessory cells in addition to the helper lymphocyte clones, it is not possible to draw conclusions about the cellular source of the haematopoietic factors from our results. It therefore remains for future experiments to determine the identity of the accessory cells, the cellular source of the factors, and the nature of the I-A-restricted interaction.

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Regulation by angiotensin II of its receptors in resistance blood vessels

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The sensitivity of blood vessels to the vasoconstrictor effects of the hormone angiotensin II appears to be modulated by the activity of the renin–angiotensin system. Elevation of circulating angiotensin II levels by sodium depletion or renal artery stenosis is associated with a diminished pressor response to infused angiotensin II (refs 1–3). Conversely, the vasoconstrictor response to the hormone is enhanced when endogenous angiotensin II levels are reduced by sodium loading^{1,4} or nephrectomy⁵. The mechanisms of these varying effects are not known, but physiological and pharmacological experiments suggest involvement of the vascular smooth muscle receptor for angiotensin II (refs 5–8). Modification of the interaction between angiotensin II and its vascular receptor, resulting in altered responsiveness to the hormone, could occur either via 'prior occupancy' of receptors by elevated levels of endogenous angiotensin II resulting in fewer free receptors available to respond to circulating angiotensin II (ref. 5), or, elevated levels of angiotensin II could result in a decrease in receptor affinity for the hormone⁷ or a decrease in total receptor number in the vascular smooth muscle cell⁸. We now report the first direct evidence, by radioligand binding assay, that angiotensin II regulates the number of its own receptors in resistance vasculature.

We have recently characterized, in a particulate fraction from rat mesenteric arteries, ¹²⁵I-angiotensin II binding sites which have the high affinity (equilibrium dissociation constant, $K_d = 0.91$ nM), low capacity (54 fmol per mg protein), and specificity expected of the physiological angiotensin II receptor⁹. In the present study we have used this system to characterize the

vascular angiotensin II receptor in rats in which circulating angiotensin II levels were normal, chronically elevated in response to sodium depletion¹⁰, or depressed by inhibition of angiotensin I converting enzyme¹¹.

Male Sprague–Dawley rats (300–325 g) were obtained from Charles River Breeding Labs. Control rats ($n = 160$) were maintained on a normal sodium diet (0.29% Na; Ralston Purina) and tap water. Sodium-depleted rats ($n = 160$) were fed a low-sodium diet (<0.05% Na; Hartroft Formula, ICN Life Sciences Group) and distilled water for one to three weeks. A third group of rats ($n = 40$) was also fed the low-sodium diet and distilled water for three weeks, but during the final three days of the diet, the angiotensin converting enzyme inhibitor SQ-14,225 (2-D-methyl-3-mercaptopropanoyl-L-proline; Squibb), 100 mg kg⁻¹ day⁻¹, was added to the drinking water. At weekly intervals, 40 control and 40 sodium-depleted rats were killed and plasma renin activity¹² and ¹²⁵I-angiotensin II binding to mesenteric artery particulate fractions determined as previously described⁹. Similar assays were performed in the sodium-depleted, SQ-14,225-treated rats following the third day of drug administration. Mesenteric artery ¹²⁵I-angiotensin II binding site capacity and affinity were estimated by Scatchard analysis of binding data. Statistical significance was determined by unpaired, 2-tailed *t*-test, comparing the results of each treated group with those of the simultaneous controls.

In control rats, plasma renin activity averaged 3.1 ± 0.3 (s.e.m.) ng ml⁻¹ h⁻¹, and mesenteric artery particulate fractions contained 56.9 ± 1.2 fmol per mg protein of angiotensin II receptors. Neither plasma renin activity nor angiotensin II receptor density varied significantly during the three weeks on the normal sodium diet. In rats maintained on the low-sodium diet alone, plasma renin activity was increased significantly at one week, and by three weeks had reached 5.2 ± 0.5 ng ml⁻¹ h⁻¹ ($P < 0.05$ compared to control, see Fig. 1). This activation of the renin–angiotensin II system was associated with a significant decrease in ¹²⁵I-angiotensin II binding capacity in mesenteric artery particulate fractions ($P < 0.01$ compared to control, weeks 1–3; see Fig. 1). After three weeks of sodium depletion, ¹²⁵I-angiotensin II binding capacity was reduced to 76% of control (41.5 ± 3.3 vs 54.3 ± 3.8 fmol mg⁻¹). In the rats given the

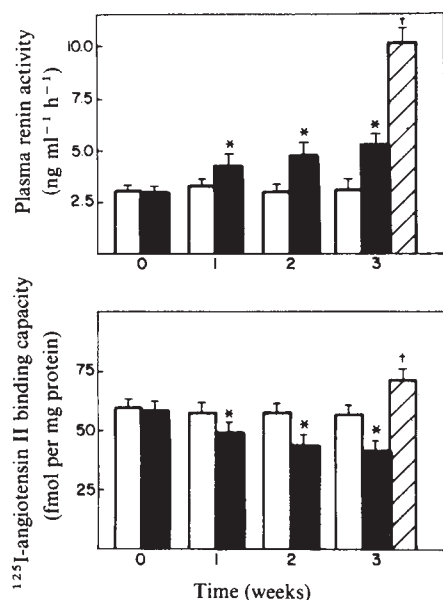


Fig. 1 Changes in plasma renin activity (upper panel) and ¹²⁵I-angiotensin II binding capacity of mesenteric artery particulate fractions (lower panel) at weekly intervals in rats fed a normal sodium diet (open bars), a sodium-deficient diet alone (solid bars), and a sodium-deficient diet plus 3 days of converting enzyme inhibitor SQ-14,225 (striped bars). Values represent mean $\pm$ s.e.m. of four experiments. For each experiment, ten control and ten treated rats were decapitated 15 min after the intraperitoneal administration of heparin, 100 U, and blood collected into iced Vacutainer® tubes containing EDTA for measurement of plasma renin activity¹². Particulate fractions from mesenteric artery homogenates were prepared from the same animals, as previously described⁹. ¹²⁵I-angiotensin II binding was determined by incubating aliquots of the particulate fraction (80–100 μ g protein) with 0.1–2.0 nM concentrations of ¹²⁵I-angiotensin II (specific activity 900–1,400 Ci mmol⁻¹) in 0.20 ml assay buffer (50 mM Tris, 5 mM MgCl₂, 0.25% bovine serum albumin, pH 7.2). After steady-state binding was achieved (90 min at 22 °C), bound and free radioactivity were separated by rapid filtration through Whatman GF/C filters and trapped radioactivity counted in a well-type gamma counter (Searle). Specific binding, defined as (total binding)–(binding in the presence of 1 μ M unlabelled angiotensin II), averaged 90% of total counts bound. * = $P < 0.05$ compared to simultaneous controls (normal diet). † = $P < 0.01$ compared to both control and sodium-deficient diet groups.

converting enzyme inhibitor SQ-14,225, during the final three days of the three-week sodium-deficient diet, plasma renin activity was significantly higher, 10.1 ± 0.6 ng ml⁻¹ h⁻¹ ($P < 0.01$ compared to third week levels in both control rats and rats sodium depleted only). The inhibition of angiotensin II formation in this group of rats, however, resulted in a significant increase in mesenteric artery ¹²⁵I-angiotensin II binding capacity, to 69.0 ± 4.3 fmol mg⁻¹ (an increase of 66% compared to the third week levels in rats sodium depleted only, $P < 0.01$, and of 27% compared to control rats, $P < 0.01$). The changes in ¹²⁵I-angiotensin II binding capacity produced by sodium depletion or converting enzyme inhibition were not accompanied by a significant change in receptor affinity (Fig. 2).

Our results suggest that the number of angiotensin II receptors in rat mesenteric arteries responds to circulating angiotensin II levels. Increased levels of hormone induced by sodium depletion resulted in a significant decrease in receptor number, whereas inhibition of angiotensin II formation by SQ-14,225 had the opposite effect. That the observed changes in receptor number correlated directly with variations in circulating angiotensin II levels is supported by the following facts: (1) ¹²⁵I-angiotensin II binding to mesenteric artery particulate fractions is independent of sodium and potassium concentrations from 0 to 100 mM (ref. 9); changes in intra- or extracellular electrolyte composition during sodium deprivation therefore could not affect the binding assay used; (2) ¹²⁵I-angiotensin II binding is likewise unaffected by bradykinin concentrations as high as 10^{-5} M (ref. 9); the increase in binding capacity following

SQ-14,225 administration thus cannot be attributed to an accumulation of endogenous bradykinin resulting from SQ-14,225 inhibition of vascular kininase II activity¹³; (3) renin itself does not appear to play a role, as the increase in plasma renin activity in sodium-depleted rats was associated with a decrease in receptor number, while an even greater increase in plasma renin activity in rats sodium depleted and given SQ-14,225 (due to loss of feedback inhibition of renin release by endogenous angiotensin II (ref. 11)) was accompanied by an increase in receptor number; and finally, (4) an artefactual change in apparent receptor binding capacity due to diet-induced changes in cellular membranes is excluded because both groups of treated rats were maintained on the same low-sodium diet, yet had opposite changes in ¹²⁵I-angiotensin II binding capacity.

A potential explanation for the results observed in these studies is that receptor-associated endogenous angiotensin II carried over into the *in vitro* binding assay could account for the decrease in binding capacity observed with sodium depletion. To provide insight into this possibility, we perfused the mesenteric arteries of control rats *in situ* for 15 min with supra-physiological (2 nM) concentrations of ³H-angiotensin II, a radioligand possessing the full biological activity of the native hormone¹⁴, and followed the amount of radioactivity remaining associated with the tissue during the usual preparation of the particulate fraction. As shown in Table 1, immediately following *in situ* perfusion, 68.4 fmol of mesenteric artery receptors were occupied by ³H-angiotensin II, yet less than 2% of the total radioactivity initially present, and essentially none of that which was specifically associated with receptors, was still detectable in

Table 1 *In vivo* labelling of mesenteric artery angiotensin II receptors by perfusion with ³H-angiotensin II

Perfusate	Crude homogenate		Particulate fraction		Total protein content (mg)
	Total radio-activity* (d.p.m.)	³ H-angiotensin II (fmol)	Total radio-activity (d.p.m.)	³ H-angiotensin II (fmol)	
³ H-angiotensin II	14,600	257.0	122	2.1	1.44
³ H-angiotensin II + antagonist	10,710	188.6	194	3.4	1.40
(Difference)	(3,890)	(68.4)	(0)	(0)	

The mesenteric arteries of three ether-anaesthetized rats (normal diet) were perfused *in situ* at a rate of 0.38 ml min⁻¹ with Krebs-Ringer phosphate buffer (pH 7.4) for 30 min at 38 °C. During the final 15 min of perfusion, ³H-angiotensin II (specific activity 25.6 Ci mmol⁻¹; NEN) was added to the perfusate at a final concentration of 2 nM. At the end of the perfusion period, the mesenteric arteries from all three rats were excised and washed in ice-cold phosphate-buffered saline (pH 7.4). The combined vessels then were homogenized in 10.0 ml ice-cold 0.25 M sucrose as described in ref. 9. An aliquot of this homogenate (1.0 ml) was counted in a liquid scintillation counter (Beckman) to determine the total amount of radioactivity initially present in the vessels from the three rats ('crude homogenate'); the remaining 9.0 ml of the homogenate were then used to prepare a particulate fraction in the same manner as for the radioligand binding assay. The resultant particulate fraction from the three rats (104,000g pellet) was resuspended in 0.5 ml water; duplicate 50- μ l aliquots were taken to measure total protein content (Lowry method) and the remaining 0.4 ml were counted as above to determine the amount of radioactivity still present in the purified particulate fraction. To determine what fraction of the radioactivity observed at each of these steps represented ³H-angiotensin II specifically associated with receptor, identical experiments were repeated in three additional rats including the angiotensin II antagonist (Sar¹, Ile⁸)-angiotensin II (1 μ M) in the perfusate for the entire 30-min perfusion period (³H-angiotensin II + antagonist). As this dose of the competitive antagonist is sufficient to block the binding of 2 nM angiotensin II to vascular receptors completely^{9,15}, the radioactivity present in the tissue from these animals presumably reflects ³H-angiotensin II nonspecifically bound or trapped in extracellular fluid. The difference in the amounts of ³H-angiotensin II present in the crude homogenates of the two groups (68.4 fmol) therefore represents ³H-angiotensin II specifically bound to receptors. On the basis of an average ¹²⁵I-angiotensin II binding capacity of 56.9 fmol per mg protein in the particulate fraction from control animals (Fig. 1), and the total protein contents of the particulate fractions in this experiment (1.40–1.44 mg), the most conservative estimate of the total number of angiotensin II receptors in each of the crude homogenates is ≈ 81 fmol. Thus, the observed value of 68.4 fmol ³H-angiotensin II specifically bound is not excessive. None of this specifically bound ³H-angiotensin II was detected in the final particulate fraction.

* Corrected for counter efficiency and quenching.

the final particulate fraction. Thus, in the experimental conditions of this study, any angiotensin II bound to receptor, after relatively brief exposure *in vivo*, seems to be readily dissociable. Endogenous angiotensin II bound in this state therefore could not account for the observed changes in binding capacity.

The apparent inverse relationship between ambient hormone concentration and angiotensin II receptor density in vascular smooth muscle is similar to that observed with other peptide hormones, such as insulin¹⁶, glucagon¹⁷ and growth hormone¹⁸. In contrast, angiotensin II seems to regulate its receptors in the adrenal gland in a different manner¹⁹. In this tissue, angiotensin II-stimulated aldosterone production is potentiated when circulating levels of angiotensin II are chronically elevated, as during sodium depletion^{20,21}; *in vitro* binding studies have shown that this increased sensitivity is accompanied by an increase in adrenal angiotensin II receptors^{21,22}. Although the regulation by endogenous angiotensin II concentrations of end-organ responsiveness of adrenal and vascular tissues to angiotensin II is directionally opposite, both effects appear to be mediated, at least in part, by hormone-induced changes in receptor number.

Previous direct studies of the regulation of angiotensin II receptors in smooth muscle have used uterine membrane fractions. These studies have yielded conflicting results. Bilateral nephrectomy has been shown to increase both the contractile response of uterine smooth muscle to angiotensin II (ref. 23) and the angiotensin II binding capacity of uterine membranes²⁴. The effects of sodium depletion on uterine smooth muscle, however, are contradictory. While Aguilera *et al.*²¹ found that sodium depletion for seven days in rats decreased uterine angiotensin II-binding capacity by 40%, Devynck *et al.*²² reported that sodium depletion for four weeks resulted in an increase in binding capacity. Although the reasons for this discrepancy are not clear, the difficulties in extrapolating binding data from the uterus, a tissue which does not respond to physiological concentrations of angiotensin II, to vascular smooth muscle have been discussed²².

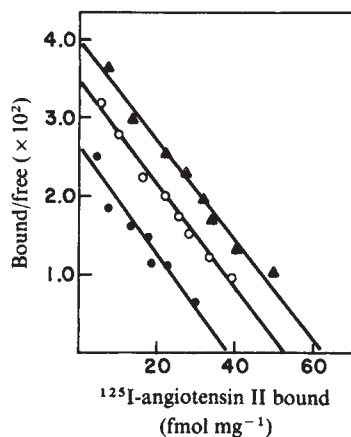


Fig. 2 Representative Scatchard plots of ¹²⁵I-angiotensin II binding to mesenteric artery particulate fractions from control rats (○), rats sodium depleted for 3 weeks (●), and from sodium-depleted rats given SQ-14,225 (▲). Particulate fractions from ten rats were used in each experiment. Mean equilibrium dissociation constants (K_d) for control (1.04 ± 0.19 nM), sodium-depleted (0.79 ± 0.07 nM), and SQ-14,225-treated (0.94 ± 0.12 nM) groups were not significantly different.

The changes in vascular angiotensin II receptors directly demonstrated in this study, during chronic sodium depletion and converting enzyme inhibition, are consistent with the hypothesis that receptor regulation can modulate vascular responsiveness to angiotensin II. At the cellular level several possible mechanisms could contribute to these hormone-induced alterations in receptor number. As the *in vivo* receptor labelling

experiment indicates, readily reversible binding of hormone to its receptor, *per se*, should not directly influence binding capacity. However, association of hormone with a high-affinity, slowly reversible state of its receptor could result in a transient decrease in binding capacity, through reduction in the number of available receptors. This form of receptor occupancy, if accompanied by uncoupling from secondary biochemical events, might lead to diminished physiological responsiveness. Alternatively, fluctuations in ambient hormone concentration might influence the rates of receptor degradation and/or synthesis, thus changing total receptor number. The relative contributions of these two mechanisms cannot be determined by current techniques *in vivo*. However, preliminary data from our laboratory, in cultured vascular smooth muscle cells suggests that both processes may be involved in regulating the number of angiotensin II receptors available for interaction with hormone. Exposure of cultured rat mesenteric artery smooth muscle cells to 3×10^{-8} M angiotensin II for up to 6 h results in a time-dependent decrease in ¹²⁵I-angiotensin II binding capacity. The decrease in binding capacity resulting from brief exposure (5 min) is completely reversible within 30 min after removal of angiotensin II from the medium. With longer periods of exposure to angiotensin II (up to 6 h), ¹²⁵I-angiotensin II binding capacity decreases progressively and recovery requires 4–5 h. Unlike the recovery after short angiotensin II exposure, complete recovery of binding capacity after 6 h of angiotensin II exposure requires new protein synthesis, implying that receptor degradation has occurred. Thus, readily reversible alterations of the angiotensin II receptor may contribute to the relatively rapid changes in vascular responsiveness seen *in vivo* during acute manipulations of the renin-angiotensin system^{25,26}. On the other hand, chronic changes in the activity of the renin-angiotensin system may be associated with changes in the kinetics of receptor turnover in vascular smooth muscle cells. These forms of hormone-induced receptor regulation may be important determinants of vascular responsiveness to the vasoconstrictor effects of angiotensin II.

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Regulation of non-muscle myosin assembly by calmodulin-dependent light chain kinase

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The presence of actin and myosin in non-muscle cells suggests that they may be involved in a wide range of cellular contractile activities¹. The generally accepted view² is that interaction between actin and myosin in these cells³⁻⁵ and in vertebrate smooth muscle^{6,7}, is regulated by the level of phosphorylation of the 20,000-molecular weight (MW) light chain. In the absence of calcium, this light chain is not phosphorylated and the myosin cannot interact with actin. Calcium activates a specific calmodulin-dependent kinase⁸⁻¹¹ which phosphorylates the light chain, initiating actin-myosin interaction. Although most studies on the role of phosphorylation have concentrated on the regulation of actin-activated myosin Mg-ATPase activity, phosphorylation of the light chain also seems to control the assembly of smooth muscle myosin into filaments^{12,13}. Using purified smooth muscle light chain kinase, we have confirmed this observation. We report here studies of myosins isolated from the two non-muscle sources, thymus cells and platelets. We observed that these myosins are assembled into filaments at physiological ionic strength and Mg-ATP concentrations, only when the 20,000-MW light chain is phosphorylated.

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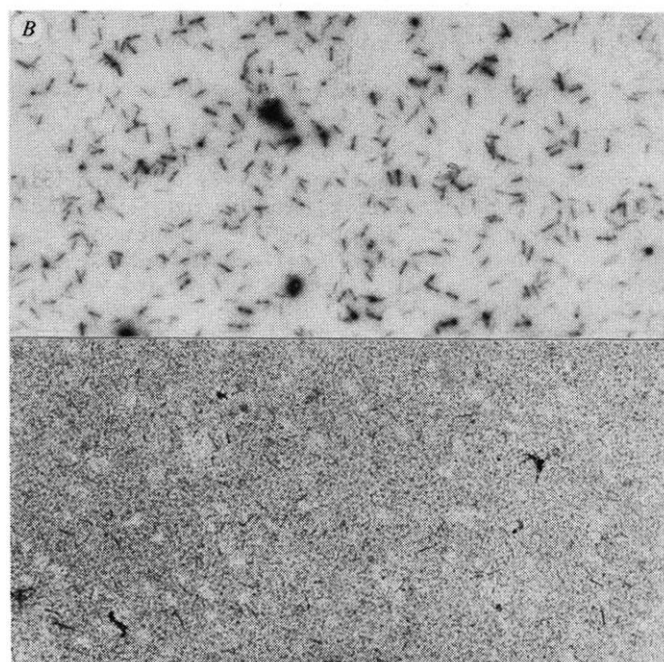
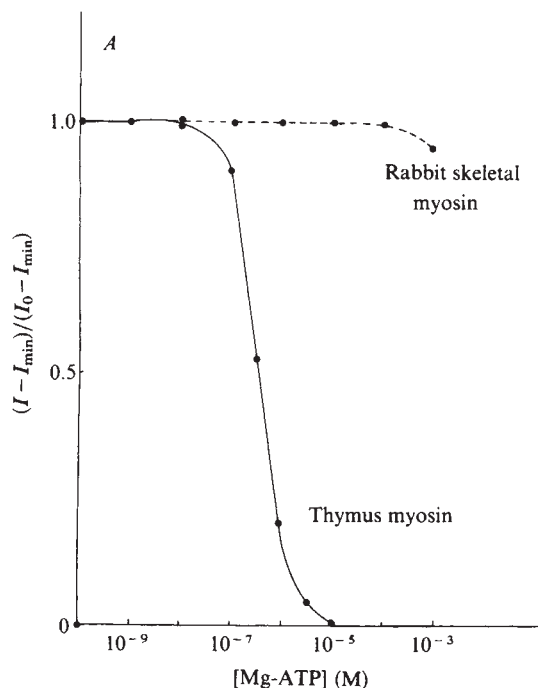


Fig. 1 The effect of Mg-ATP on thymus myosin. **A**, Thymus myosin was prepared essentially by the method of Pollard *et al.*¹⁸ for platelet myosin. Samples of myosin (0.5 mg ml⁻¹) were dialysed into 0.15 M KCl, 10 mM MgCl₂, 1 mM EGTA, 0.1 mM DTT, 25 mM imidazole pH 7.0. Turbidity at $\lambda = 340$ nm was recorded in a Perkin Elmer model 551 double beam spectrophotometer at 20 °C. Mg-ATP concentration was varied by the addition of stock solutions. To correct for the effects of dilution, an equal aliquot of buffer was added to control samples of myosin. Background turbidity was corrected by plotting $(I - I_{\min}) / (I_0 - I_{\min})$. I is turbidity measured at 340 nm, I_0 the turbidity in the absence of Mg-ATP, and $I_{\min}$ the minimal level of turbidity attained during the experiment. Typical values are $I_0 = 0.4$ and $I_{\min} = 0.15$. It must be stressed that turbidity measurements should always be used in conjunction with electron microscope observations so as to 'estimate' the degree of filament assembly-disassembly. **B**, Electron micrographs of thymus myosin before (top) and after (bottom) addition of 50 μ M Mg-ATP. Specimens were prepared by placing 10 μ l of the myosin solution to be examined on to carbon-coated 400-mesh grids, allowing the drop to stand for exactly 30 s, then washing with 13 drops of buffer (-ATP) and 10 drops of 1% uranyl acetate. Micrographs were examined on a Philips EM400 operated at 80 kV. $\times 5,000$.

We initially compared the stability of myosin filaments prepared from non-phosphorylated calf thymus myosin and rabbit skeletal myosin as a function of the Mg-ATP concentration (Fig. 1). The turbidity measurements and electron micrographs show that the filaments from thymus myosin (bipolar filaments, average length $3,380 \pm 560$ Å) are disassembled completely at a molar ratio of less than 5 mol Mg-ATP per mol myosin, suggesting that Mg-ATP acts at an approximately stoichiometric ratio. Similar results were obtained with myosins isolated from pig platelets and chicken gizzard muscles. These results differ from those of earlier work¹⁴ which indicated that myosin isolated from human platelets formed short bipolar filaments which were stable in a wide range of conditions, including the presence or absence of Mg-ATP at physiological ionic strength.

Filaments prepared from phosphorylated thymus or gizzard myosins were stable at millimolar concentrations of Mg-ATP. Similarly, thymus and gizzard myosin rod subfragments (the tail fragment which contains the self-assembly properties of the myosin) formed filaments which were not disassembled at the Mg-ATP concentrations used in these experiments (up to 10 mM). Mg-pyrophosphate would not substitute for Mg-ATP in disassembling non-phosphorylated thymus or gizzard myosin filaments. These observations suggest that Mg-ATP acts at a specific high-affinity binding site in the subfragment-1 (myosin head) portion of the molecule, which may be the Mg-ATPase active site.

Myosins isolated from rabbit and scallop striated muscles form filaments which are stable at millimolar concentrations of Mg-ATP. In this case, the lack of effect of Mg-ATP must be due to differences in the subunits of these myosins compared with those of vertebrate smooth muscle and non-muscle myosins. These experiments were carried out in conditions roughly comparable to those that are expected to exist inside a 'resting' cell, namely 0.15 M KCl, 1 mM Mg-ATP and the absence of Ca²⁺. We therefore predict that 'in vivo', the myosin in resting

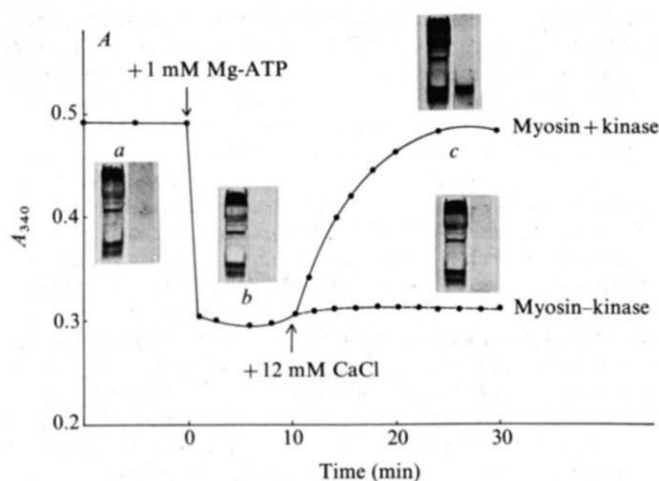


Fig. 2 Induction of thymus myosin filament assembly by phosphorylation of the 20,000-MW, light chain. **A**, Non-phosphorylated myosin filaments (0.46 mg ml^{-1}) were incubated with $25 \mu\text{g ml}^{-1}$ brain calmodulin in 0.15 M KCl , 10 mM MgCl_2 , 1 mM EGTA , 0.1 mM DTT and $25 \text{ mM imidazole pH 7.0}$. Myosin light chain kinase purified from either chicken gizzard or thymus by calmodulin affinity chromatography¹⁹ was included where indicated. Turbidity was recorded as in Fig. 1. $1 \text{ mM } [\gamma^{32}\text{P}]\text{-ATP}$ was added and samples were taken at time points *b* and *c* and the level of myosin phosphorylation determined (Table 1). The specificity of phosphate incorporation into the myosin 20,000-MW light chain was determined by SDS-polyacrylamide gel electrophoresis (PAGE) and subsequent autoradiography (shown in the insets are the 10% acrylamide/SDS gels together with their autoradiograms). The specificity of incorporation was further checked by 8 M urea-PAGE (data not shown). The addition of Mg-ATP caused a rapid drop in turbidity, which remained low until the kinase was activated by addition of CaCl_2 to a concentration of $2 \times 10^{-4} \text{ M}$ free Ca^{2+} , whereupon a steady increase in turbidity was observed to accompany light chain phosphorylation. In the absence of kinase, there was no light chain phosphorylation or turbidity increase on addition of CaCl_2 . **B**, Electron micrographs of thymus myosin taken at three critical time points, *a*, *b* and *c* in the assembly assay. Specimens prepared as described in Fig. 1. Top, non-phosphorylated myosin filaments in the absence of ATP; middle, after addition of Mg-ATP to 1 mM , myosin filaments completely disappear, and in their place, a high concentration of dissolved protein is observed on the grid; bottom, after addition of calcium, filaments reappear. $\times 5,000$.

non-muscle and smooth muscle cells exists in a disassembled state, whereas in relaxed vertebrate and invertebrate striated muscles, myosin retains its filamentous organization, in agreement with direct observations on relaxed skeletal muscles¹⁵.

Table 1 Correlation of light chain phosphorylation and actin-activated Mg-ATPase of thymus myosin with filament assembly

Time point during 'assembly assay' (see Fig. 2A)	-Kinase mol phosphate per mol light chain	Mg- ATPase (nmol per min per mg)	+Kinase mol phosphate per mol light chain	Mg- ATPase (nmol per min per mg)
1 mM EGTA (Fig. 2b)	0.1	4.0	0.25	7.0
$2 \times 10^{-4} \text{ M Ca}^{2+}$ (Fig. 2c)	0.2	4.0	0.75	26.0

Light chain phosphorylation and actin-activated Mg-ATPase activities were determined in conditions identical to those used in the assembly assay at the time points marked *b* and *c* in Fig. 2A. The conditions were 25°C , 0.15 M KCl , 10 mM MgCl_2 , $0.1 \text{ mM dithiothreitol (DTT)}$ and $25 \text{ mM imidazole pH 7.0}$. At time point *b*, 1 mM EGTA was present in the buffer, and at time point *c*, 1 mM EGTA and 1.2 mM CaCl_2 were present. Myosin phosphorylation was determined by a Millipore filtration method similar to that of Sherry *et al.*⁷. Mg-ATPase activity was determined according to the method of Seals *et al.*¹⁷, after addition of skeletal actin to a 10-fold weight excess over myosin. At time point *c* the kinase had phosphorylated the myosin light chain, promoting interaction with actin in addition to inducing myosin filament assembly.

Phosphorylation of the 20,000-MW light chain of thymus myosin, disassembled in the presence of Mg-ATP, induces myosin filament assembly. Using calmodulin-dependent light chain kinase purified from either chicken gizzard or calf thymus cells, we have monitored the effect of phosphorylation on the assembly of thymus myosin filaments (Fig. 2). Addition of 1 mM Mg-ATP to non-phosphorylated myosin filaments incubated in the presence of light chain kinase, calmodulin and EGTA, caused a rapid drop in turbidity (Fig. 2A). In the electron microscope, the short bipolar filaments which were present before ATP addition are now absent (Fig. 2B). The myosin filaments remained disassembled until the kinase-calmodulin complex was activated by the addition of calcium. A steady increase in turbidity and the appearance of bipolar filaments were then observed (average length $5,000 \pm 640 \text{ \AA}$). The reassembly of the myosin filaments accompanies the phosphorylation of the 20,000-MW light chain (Table 1). In the absence of kinase or calcium, there was no light chain phosphorylation or filament formation. We have observed similar effects with human platelet myosin. These results raise the question of whether phosphorylation regulates myosin filament assembly in all non-muscle cells. Transient assembly of smooth muscle myosin, regulated by light chain phosphorylation, has also been suggested by Suzuki *et al.*¹². Our results using chicken gizzard myosin are in total agreement with their data. We believe that these findings may be very relevant to the unresolved controversy surrounding the state of myosin in 'relaxed' smooth muscles¹⁶.

Our results suggest that the assembly of myosin filaments may be a crucial step in the pathway leading to non-muscle myosin

activation by calmodulin-dependent light chain kinase. However, is it the only step involved in activation, for what contribution does filament assembly make to the observed stimulation of the actin-activated myosin Mg-ATPase activity controlled by light chain phosphorylation (Table 1)? Experiments are in progress to answer this question. Several other important questions remain to be resolved. If myosin filament assembly mediated by phosphorylation is involved in activation, one would predict that relaxation is controlled by dephosphorylation of the myosin and filament disassembly. Experiments are being carried out to test whether the process of filament assembly can be reversed by dephosphorylation of the myosin. How does phosphorylation of a light chain associated with the subfragment-1 region of the myosin modulate the assembly of myosin into filaments, which is generally assumed to be due to interactions between the light meromyosin (LMM)

portions of the molecules? To answer this question, we need to know the position of the light chain on the myosin head, the changes that occur when it is phosphorylated or dephosphorylated, and how these changes affect the packing of the myosin molecules in the filament.

In conclusion, we propose that in 'resting' vertebrate non-muscle cells, myosin filaments are absent and are formed transiently during periods of contractile activity. Our observation that the activation process—phosphorylation—generates bipolar myosin filaments provides circumstantial evidence that some type of sliding-filament interaction¹⁵ between actin and myosin may operate in vertebrate non-muscle cells.

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Endogenous electric field around muscle fibres depends on the Na⁺–K⁺ pump

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We describe here experiments which reveal a new physiological specialization in the endplate (synaptic) region of skeletal muscle fibres. Using a vibrating microelectrode¹ which can detect small currents flowing in extracellular fluid, we have found that the membrane in the endplate region behaves as though a steady positive current is generated in this location. Current re-enters the fibre in the extrajunctional region. Further experiments show that this current is dependent on the activity of the sodium pump. The electric field created by this current may be important for long-term interactions between muscle and nerve.

Most experiments to be described were performed on small (lumbrical and flexor digitorum brevis) muscles of the rat hind foot, although similar results were obtained in a few experiments with mouse soleus and frog sartorius muscles. Experiments were performed *in vitro* at room temperature (23 °C). Muscles were bathed in Krebs solution buffered to pH 7.4 with bicarbonate/CO₂ or HEPES.

The vibrating microelectrode works as follows: a metal-filled glass micropipette with a platinum ball (10–15-μm diameter) electroplated on its tip is attached to a piezoelectric bender, which is driven at a constant frequency (about 260 Hz). Excursion

distance of the electrode tip is about 35 μm. The input signal is led to a lock-in amplifier (Princeton Applied Research), which filters (with bandpass equal to the vibration frequency), rectifies and then averages the signal over many cycles to reduce noise further (response time constant was 1–10 s). Thus, the output provides a measure of the peak-to-peak potential difference between the electrode tip at its excursion limits. Each electrode is calibrated with a known current source. Peak-to-peak input voltages were calculated by multiplying d.c. output values by 2/0.707; all values presented in text and figures are peak-to-peak. Potential differences of 0.1 μV can be readily detected. Krebs solution resistivity is 75 Ω cm; thus 0.1 μV corresponds to a current density of about 1 μA cm⁻². The electrode vibration was oriented perpendicular to the long axis of the muscle; thus, when adjacent to a muscle fibre, it provided a measure of membrane current at that point. Baseline values were obtained with the electrode vibrating far (several mm) from the muscle.

The spatial distribution of current was mapped along the lateral margin of whole muscles (Fig. 1a). Outward current was detected near the middle of the muscle, and the maximum outward current was invariably located at the endplate zone, which was identified by subsequent staining for acetylcholinesterase. In the flanking regions, membrane current reversed direction and flowed inward (Fig. 1a; b, filled circles). This pattern is drawn schematically in Fig. 1c, which also illustrates relative dimensions of the electrode and fibre.

To gain further information about the spatial distribution of current sources and sinks, experiments were repeated on single isolated muscle fibres. Flexor digitorum brevis muscles were incubated in Krebs containing 0.1–0.3% collagenase for 2–3 h, and then triturated to release hundreds of single, intact fibres². The current pattern around single fibres was like that obtained for whole muscles (see Fig. 1b, open circles). The peak outward current occurred at the endplate, which could be visually identified on the living cells. In some cases it appeared that the nerve terminals had been completely removed by the isolation procedure. Such fibres still generated an outward current in the endplate region; this identifies the muscle membrane as a source of the current. The amplitude of the signal fell off rapidly as the electrode was moved away from the muscle fibre, becoming undetectable when the electrode was a few hundred μm from the fibre. This suggests that, in the whole muscle experiments,

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only the fibres near the surface contributed to the recorded signal. Maximal outward current at the membrane was estimated to be about $10 \mu\text{A cm}^{-2}$, and integration of the current profile around the circumference of the fibre gave a value of total outward current of about 1 nA. In a muscle fibre with an input resistance of $0.5 \text{ M}\Omega$, this should produce a hyperpolarization of about 0.5 mV.

Five different kinds of experiments suggest a role for the sodium-potassium pump in the generation of the outward current. In each experiment, whole muscles were used and, after initial spatial mapping, all measurements were made at the site of the maximum outward current density (endplate zone). (1) Effect of potassium removal: when Krebs solution without potassium was perfused through the chamber, the outward current was abolished within 15–30 s (Fig. 2a). The effect was readily reversible. The rapidity of these effects suggests again that only fibres near the surface of the muscle contributed to the recorded signal. (2) Effects of temperature: perfusing the chamber with ice-cold Krebs solution caused the current to decrease greatly. Conversely, the signal increased in amplitude when warmed Krebs solution (37°C) was infused. These results

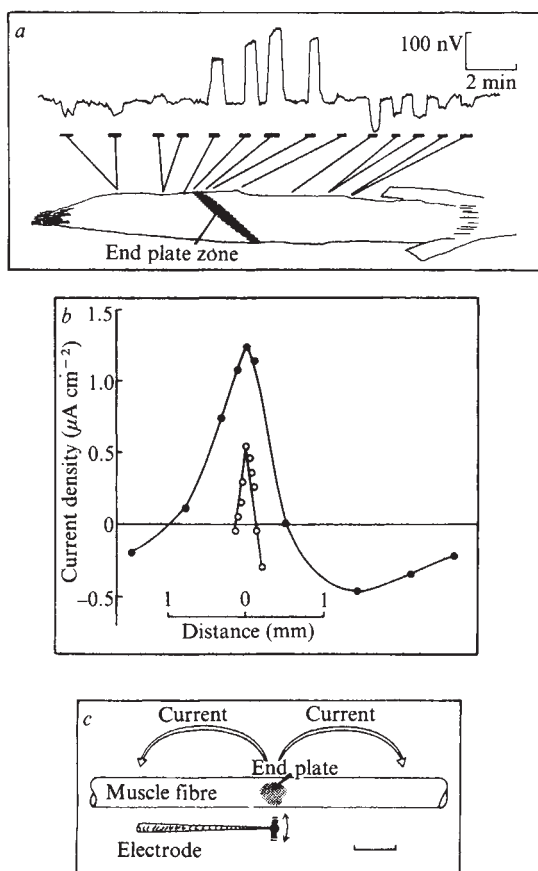


Fig. 1 a, Current pattern around whole muscle. The electrode, vibrating continuously, was alternately moved close to the muscle (marked by bars) and then away to a remote reference position in the bath. Recording positions near the muscle are shown by lines connecting the bars to the drawing of the muscle; the stippled area in the drawing marks the endplate zone. The current was largest when the electrode was at the endplate zone, and reversed sign in the flanking regions. Upward deflection represents outward current. The electrode was vibrated perpendicular to the long axis of the muscle (see c). b, Amplitude of the current (recorded near the muscle) is plotted as a function of distance from the endplate region. Results from $\bullet$, an experiment on a whole muscle, and $\circ$, an isolated muscle fibre. c, Schematic diagram of a single muscle fibre depicting endogenous current pattern. The electrode tip and muscle fibre are drawn to illustrate approximate scale (bar = $50 \mu\text{m}$).

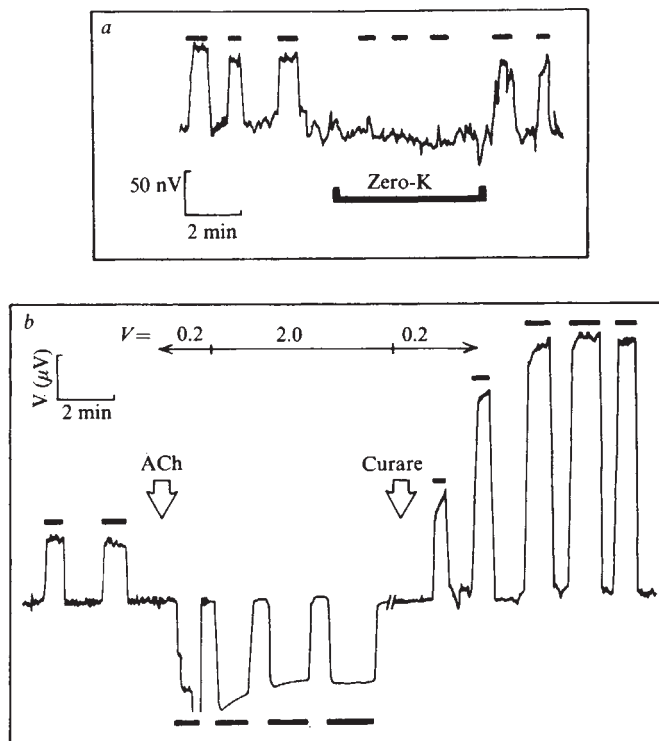


Fig. 2 a, Continuous recording obtained during an experiment to test the effect of removal of external potassium ions. During the times marked by the bars, the electrode was close to the muscle, at the endplate zone. Note that the endogenous current was abolished in zero-K Krebs (upward deflection = outward current). b, Experiment to test the effect of 'sodium loading' of the endplate region. Recordings near the muscle (marked by bars), were made at the endplate region. After the first two control readings, the chamber was perfused with Krebs containing ACh (plus prostigmine), and the current reversed sign, becoming inward and very large (note 10-fold gain change). When the chamber was later perfused with Krebs containing curare, the current again became outward, and was about fourfold larger than control. All solutions contained tetrodotoxin; the time break represents about 20 min.

are consistent with the involvement of a pump. (3) Effect of ouabain: the cardiac glycoside ouabain is a specific inhibitor of the sodium pump. When mouse soleus muscle was superfused with Krebs solution containing 0.1 mM ouabain, the current was rapidly abolished. The effect was less clear with rat muscle; 10 mM ouabain reduced, but did not abolish the signal. Rat muscle, however, is known to be relatively resistant to ouabain³. (4) Effects of sodium replacement: external sodium was completely replaced by lithium. Lithium ions can enter muscle cells, but are not pumped out by the $\text{Na}^+\text{-K}^+$ pump. The Li solution reversibly abolished the current over a period of several minutes. This effect was probably due to a gradual reduction of internal sodium concentration, so that the $\text{Na}^+\text{-K}^+$ pump was no longer active. (5) Sodium 'loading' in the endplate region (Fig. 2b). Intracellular sodium concentration in the endplate region was increased by perfusing with Krebs solution containing acetylcholine (ACh, $5 \times 10^{-4} \text{ g ml}^{-1}$) or carbachol ($3 \times 10^{-5} \text{ g ml}^{-1}$). These substances activate ACh receptors, whose distribution is restricted to the membrane in the endplate region, and which, when activated, produce an influx of sodium ions. The presence of these drugs changed the current recorded in the endplate region from outward to an extremely large inward current, reflecting the entry of sodium ions. When the preparation was subsequently perfused with Krebs solution containing curare ($1 \times 10^{-5} \text{ g ml}^{-1}$) or α -bungarotoxin ($4 \times 10^{-6} \text{ g ml}^{-1}$) and no ACh or carbachol, the ACh receptors were blocked, and the recorded current reversed again, becoming about four times larger than normal. (Control experiments showed that curare, α -bungaro-

toxin, and tetrodotoxin by themselves had no effect on the normal outward current). The increased amplitude of the outward current is consistent with a more active Na^+ pump, due to the increased internal sodium concentration.

The results of these experiments lead to the conclusion that the steady currents which were measured are produced or maintained by the Na^+-K^+ pump. The existence of such pumps and their electrogenicity in skeletal muscle are known⁴. The new finding reported here is the non-uniform spatial distribution of membrane current sources and sinks. This must reflect either a non-uniform distribution of pumping activity (that is, pumps that are more concentrated, more active, or more electrogenic in the endplate region) or a non-uniform distribution of leakage channels, or both. One hypothesis is that there is a higher concentration of electrogenic Na^+-K^+ pump sites in the endplate region than elsewhere, reflecting the need for removal of sodium ions that enter the fibre through ACh channels during nerve-evoked excitation. However, the outward current persisted *in vitro* for several hours in the absence of nerve-evoked activity (even after ACh receptors were totally blocked by curare or α -bungarotoxin), suggesting that the currents measured in our experimental conditions were independent of fluxes through ACh channels.

Whatever the distribution of pumps and leaks, their non-uniformity produces an electric current and hence an electric field, whose focus is centred on the endplate region. It is naturally of interest to inquire whether this field might serve other functions. For instance, it has recently been shown that ACh receptor distribution on myotubes⁵ and growth of neurites in culture (ref. 6, and McCaig, Robinson and Hinkle, unpublished observations) can be affected by externally applied electric fields. This raises the possibility that the endogenous electric field reported here might be significant for long term interactions between neurones and muscle fibres. The estimated peak value of the electric field we measured around single, isolated muscle fibres is one to two orders of magnitude smaller than the fields required for effects on ACh receptor distribution or neurite growth in the studies cited above. However, those conditions and preparations were considerably different from the ones reported here. Moreover, the electric field which exists deep within an intact muscle may be larger than that measured near an isolated fibre. For instance, both the restricted volume of extracellular space and the presence of many endplates lying in register would serve to increase electric field strength.

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Active sodium transport by turtle colon via an electrogenic Na-K exchange pump

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Active sodium absorption by a variety of epithelia is abolished by ouabain, but the obligatory coupling between the movement of sodium and potassium expected from a basolateral ($\text{Na}^+ + \text{K}^+$) ATPase has not been convincingly demonstrated¹. According to the model of Koefoed-Johnsen and Ussing², the asymmetric cation selectivities of the apical and basolateral membranes prevent basolateral Na-K exchange from being expressed as opposing transmural ion flows. An additional consequence of this asymmetry is that the short-circuit current

(I_{sc}) cannot be identified with the current through the sodium-potassium pump. We used the polyene antibiotic, amphotericin-B, to reduce the resistance and the cation selectivity of the apical membrane of isolated turtle colon so that the basolateral membrane current could be dissected into two components: one through a barium-sensitive potassium channel and another which represents the current associated with ouabain-sensitive, electrogenic, Na-K exchange. Comparison of cation fluxes and short circuit current indicates that in these conditions active sodium absorption is entirely attributable to an electrogenic Na-K pump with a stoichiometry of approximately $3\text{Na}:2\text{K}$.

The presence of a basolateral membrane potassium channel was established by allowing tissues to attain a steady state I_{sc} and then subjecting them to the following sequence of manoeuvres. The steady-state I_{sc} was virtually abolished with ouabain (10^{-4} M , serosal) and then amiloride (10^{-4} M) was added to the mucosal bathing solution. Subsequently, the potassium concentration of either the mucosal or serosal bathing solution was raised to 10-30 mM by the addition of a small volume of concentrated potassium methyl sulphate or KCl. These additions had virtually no effect on I_{sc} although total tissue conductance (G_{T}) usually increased slightly, presumably due to an increase in the conductance of the paracellular shunt path^{3,4}. Figure 1 shows the results of a representative experiment in which amphotericin-B ($6\text{ }\mu\text{M}$) was added to the mucosal bathing solution in the presence of a mucosal to serosal potassium gradient (M:30 mM, S:3.0 mM). Addition of the polyene produced a prompt increase in I_{sc} and G_{T} . The steady-state I_{sc} was consistent with net cation flow from mucosa to serosa and, as indicated in the figure, was abolished by the addition of BaCl_2 (5 mM) to the serosal bath. The polyene-induced increase in I_{sc} was always consistent with the orientation of the applied potassium e.m.f.

To determine the properties of the ouabain-sensitive active cation transport mechanism, portions of isolated colon bathed by identical solutions containing 102 mM Na and 2.5 mM K were subjected to the following procedure. First amiloride (10^{-4} M) was added to the mucosal side to reduce I_{sc} to near zero

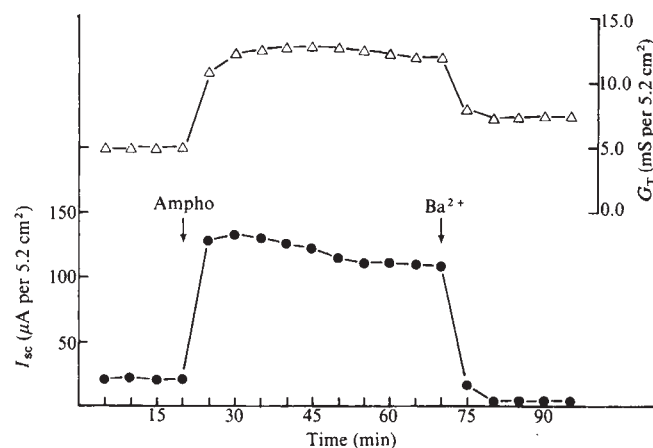


Fig. 1 Portions of stripped turtle colon were mounted in Ussing chambers (area 5.2 cm^2) and bathed on both sides by solutions containing (in mM) sodium benzene sulphonate 100, potassium bicarbonate 2.5, calcium chloride 1.0, sodium pyruvate 2.0, and glucose 5.0. Both solutions were stirred with air so that the pH at room temperature was approximately 8.4. Benzene sulphonate was substituted for chloride in an attempt to prevent cell swelling due to net salt movement into epithelial cells in the presence of the polyene⁶. The figure shows short-circuit current (●) and total tissue conductance (Δ) as a function of time. Active sodium absorption was abolished by adding ouabain (10^{-4} M) to the serosal bath. In the presence of a transmural potassium gradient (M 30 mM, S 2.5 mM) the addition of amphotericin-B ($6\text{ }\mu\text{M}$) increased I_{sc} and G_{T} . Subsequent addition of BaCl_2 (5 mM) to the serosal bath abolished I_{sc} and reduced G_{T} .

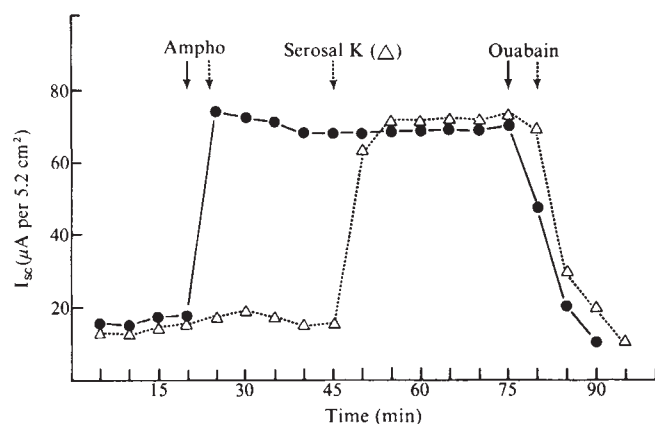


Fig. 2 Short-circuit current as a function of time for colons bathed initially by potassium-containing (●) and potassium-free (Δ) solutions. I_{SC} was inhibited with mucosal amiloride (10^{-4} M), and $BaCl_2$ (5 mM) was added to the serosal bath. Mucosal amphotericin-B (6 μ M) increased I_{SC} only in the presence of serosal potassium. Subsequent addition of potassium to the K-free tissue (Δ) resulted in a prompt increase in I_{SC} . In both tissues I_{SC} was abolished by ouabain (10^{-4} M).

and abolish sodium absorption², then $BaCl_2$ (5 mM) was added to the serosal bath to decrease basolateral potassium conductance. Figure 2 (closed circles) shows the result of a representative experiment in which amphotericin-B (6 μ M) was added to the mucosal bath. Addition of the polyene in the absence of electrochemical gradients produced a prompt increase in I_{SC} to a new steady state consistent with net positive charge movement from mucosa to serosa. The polyene-induced I_{SC} was stable for more than 60 min and was abolished by serosal ouabain (10^{-4} M).

The ouabain-sensitive, polyene-induced I_{SC} exhibited an obligatory dependence on serosal potassium. The triangles in Fig. 2 represent a tissue initially bathed on the serosal side by potassium-free, Na benzene sulphonate Ringer's. The addition of amphotericin-B had virtually no effect on I_{SC} ; however, subsequent addition of KCl (2.5 mM) to the serosal bath resulted in a prompt increase in I_{SC} to a level virtually identical to that of the control. A further increase in serosal potassium concentration (data not shown) produced an additional increase in I_{SC} suggesting that in these conditions the pump rate is limited by the supply of serosal potassium.

Figure 3 shows the results of a similar experiment designed to evaluate the dependence of the polyene-induced I_{SC} on mucosal

sodium. Both tissues were bathed initially on the serosal side by benzene sulphonate Ringer's containing 102 mM Na and 2.5 mM K; and on the mucosal side by Ringer's which was identical except that the sodium benzene sulphonate had been replaced with choline benzene sulphonate containing less than 1 mM sodium. Both mucosal solutions contained amiloride and in one tissue (closed circles) the mucosal sodium concentration was raised to 20 mM by the addition of a small volume of concentrated Na benzene sulphonate. Subsequent addition of amphotericin-B to both tissues produced a prompt increase in I_{SC} only in the tissue exposed to a sodium-containing mucosal solution. Addition of sodium to the second tissue (triangles), however, increased I_{SC} to a level comparable to the first. The amphotericin-induced I_{SC} in both tissues was abolished by ouabain.

In view of the striking dependence of the ouabain-sensitive, amphotericin-induced I_{SC} on serosal potassium and mucosal sodium we measured transmural fluxes of these ions to determine the ionic basis of the current. The results are shown in Table 1 where the amphotericin-induced increases in net sodium absorption and potassium secretion are shown along with the corresponding amphotericin-induced increases in I_{SC} . The results indicate that net Na absorption is approximately three times I_{SC} whereas net potassium secretion is about twice I_{SC} , so that the transepithelial current can be attributed to the difference between the net fluxes of the two cations. Previous studies on lipid bilayers⁵, red blood cells⁶ and isolated epithelia⁷⁻⁹ have suggested that the polyene antibiotics can form pores in natural and artificial membranes which are moderately cation selective but do not discriminate appreciably between sodium and potassium. Neilsen⁶ recently used the polyene antibiotic, filipin, to reduce the cation selectivity of the apical membrane of frog skin and obtained results consistent with the existence of a sodium-potassium exchange pump. In these experiments, however, the 'pump current' and its relation to active cation fluxes was not clearly defined.

In the presence of mucosal polyene, the transport properties of the turtle colon appear to be dominated by the properties of the basolateral membrane which must contain at least two cation transport elements: a barium-sensitive potassium conductance and an electrogenic sodium-potassium exchange pump. In the presence of serosal ouabain and mucosal polyene the turtle colon is markedly potassium selective, and this potassium

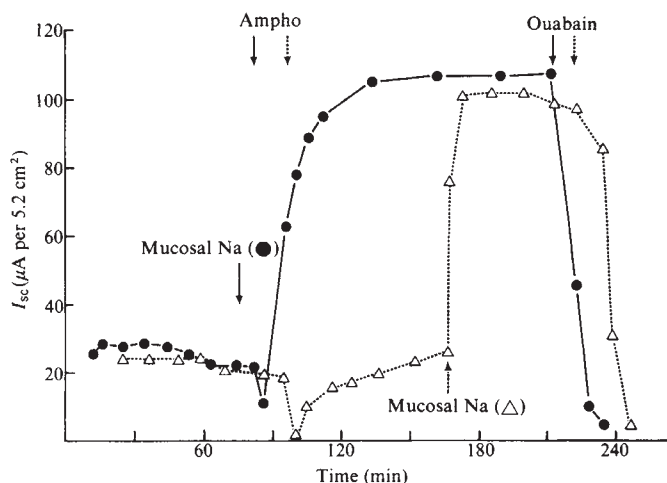


Fig. 3 Short-circuit current as a function of time for tissues bathed initially by sodium-free mucosal solutions and treated with amiloride (mucosal bath) and $BaCl_2$ (serosal bath). After the addition of sodium (20 mM) to the mucosal solution of one (●), amphotericin-B was added to the mucosal solution of both tissues. I_{SC} increased only in the presence of mucosal sodium. Subsequent addition of mucosal sodium to the Na-free tissue (Δ) resulted in a prompt increase in I_{SC} . In both tissues I_{SC} was abolished by ouabain.

Table 1 Amphotericin-induced sodium absorption, potassium secretion and short-circuit current

	ΔI_{SC} (μ equiv. $cm^{-2} h^{-1}$)	ΔJ_{net} (μ equiv. $cm^{-2} h^{-1}$)	$\Delta J_{net}/\Delta I_{SC}$	<i>n</i>
Sodium	0.67 ± 0.04	2.00 ± 0.16	3.01 ± 0.15	15
Potassium	0.57 ± 0.11	1.14 ± 0.20	2.12 ± 0.14	8

Fluxes of ^{22}Na were measured using mucosal and serosal solutions which were identical Na benzene sulphonate Ringer's in which the potassium concentration had been raised to 14 mM in order to increase I_{SC} in the presence of the polyene. The mucosal bath contained amiloride (10^{-4} M) and the serosal bath contained $BaCl_2$ (5 mM). Net sodium fluxes were obtained by comparing unidirectional fluxes from paired tissues. Preliminary experiments revealed that ^{42}K fluxes significantly underestimated net potassium flux, hence net potassium secretion was determined by using flame photometry to measure the appearance of the ion in a nominally potassium-free mucosal bathing solution which also contained amiloride (10^{-4} M). The serosal solution contained 10 mM potassium and 5 mM $BaCl_2$. Otherwise both solutions were identical sodium benzene sulphonate Ringer's. The values appearing in the table (mean $\pm$ s.e.) were corrected for the small diffusional potassium flux which could be estimated by measuring potassium appearance before the addition of the polyene.

conductance is blocked by serosal Ba^{2+} . This result is consonant with Nagel's report¹⁰ of a Ba^{2+} -sensitive potassium conductance in the basolateral membrane of isolated frog skin. Additional experiments (data not shown) indicate that serosal Ba^{2+} also reduces ^{42}K fluxes across the epithelium in similar conditions. If ouabain is omitted from the serosal bathing solution, amphotericin-B induces a short-circuit current in the absence of transmural cation gradients. This 'active' I_{SC} exhibits an obligatory dependence on mucosal sodium and serosal potassium, is abolished by ouabain, and is numerically equal to the difference between net Na absorption and potassium secretion. These observations suggest that in the presence of serosal Ba^{2+} the polyene-induced I_{SC} represents current attributable to an electrogenic sodium-potassium exchange, where the ratio of sodium to potassium is approximately $3\text{Na}:2\text{K}$. Thus, sodium absorption by the turtle colon seems to be entirely attributable to the presence, in the basolateral membrane, of a ouabain-sensitive cation exchange pump similar to that which seems to exist in human red blood cells^{11,12} and in some excitable membranes¹³.

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Light-induced changes in membrane current in cone outer segments of tiger salamander and turtle

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The peak change in membrane conductance of vertebrate photoreceptors in response to the isomerization of a single photopigment has been estimated from recordings of membrane potential to be about 8 pS in rods¹ and 6 pS in cones². For rods the estimate has been largely confirmed by Yau *et al.*³ by directly recording membrane current responses of rod outer segments to single photoisomerizations. However, no similar measurements have been reported for cone outer segments. Here, we report on direct recordings of membrane currents of single cone outer segments using an extracellular patch electrode. The technique was similar to that described by Yau *et al.*³ for toad rods. We have measured dark currents of up to 40 pA, and calculate that the conductance change resulting from a single photoisomerization is less than 1 pS.

A portion of the back of the eye of a dark-adapted larval tiger salamander, *Ambystoma tigrinum*, or turtle, *Pseudemys scripta elegans*, was placed vitreal side down on Millipore filter paper. The sclera and pigment epithelium were gently lifted off, leaving the retina, receptor side up, attached to the filter paper. A 300- μm slice of retina and filter paper was submerged in oxygenated Ringer⁴ in a superfusion chamber placed side-on under the water-immersion objective of a conventional microscope, and viewed through an IR image converter at $\times 600$ magnification. The outer segment of a single cone was drawn into a Ringer-filled electrode. The resultant high resistance seal made between the electrode interior and the base of the outer segment of a tiger salamander cone, or between the electrode and a red oil droplet of a turtle cone, enabled us to record 50-90% of the membrane current of the outer segment. Measurements of the relative spectral sensitivities of both these receptors in membrane current per incident photon were consistent with cones containing a dehydroretinal-based pigment with an absorption maximum at 620 nm (refs 5, 6). Current was recorded by a virtual ground current-to-voltage converter with a 100 M Ω feedback resistor.

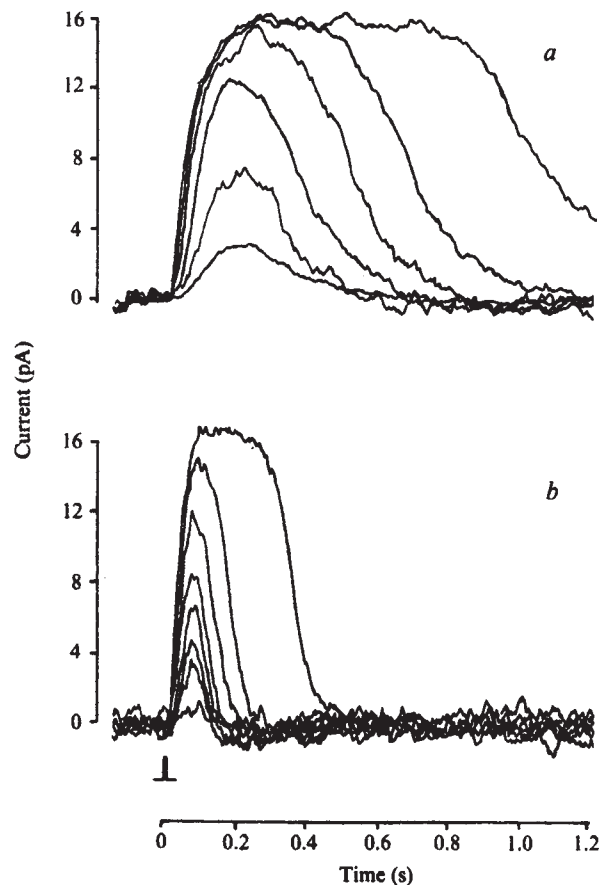


Fig. 1 *a*, Current responses of 'red' cone outer segment of tiger salamander to 10-ms flashes of diffuse white light of increasing intensity; stimulus monitor below. Downward deflection corresponds to inward membrane current, recorded with a d.c.-10 Hz bandwidth. Average number of effective photoisomerizations per flash, from bottom to top: 3.89×10^2 , 1.39×10^3 , 3.90×10^3 , 1.05×10^4 , 3.10×10^4 , 3.10×10^5 . Stimulus calibration described in Fig. 2. Each trace represents the average of one to nine light responses. The traces shown are uncorrected current records representing, on our estimation, 78% of the true current flowing across the outer segment membrane within the recording electrode. The percentage of membrane current recorded equals the difference in resistance to ground of the electrode with and without the outer segment drawn inside, divided by the resistance of the electrode with the outer segment inside. *b*, Current responses of 'red' cone outer segment of turtle to 10-ms diffuse test flash; bandwidth, d.c.-10 Hz. Number of effective photoisomerizations estimated per flash, from bottom to top: 1.5×10^2 , 6.45×10^2 , 1.22×10^3 , 1.50×10^3 , 2.07×10^3 , 4.42×10^3 , 1.60×10^4 , 1.40×10^5 . White light was used for responses 1, 3, 7 and 8; other responses were to red light of $\lambda_{\text{max}} = 610$ nm. Uncorrected current records representing 65% of the true current.

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In the dark, a current of up to 40 pA flowed into the outer segments of cones (about 30 pA averaged over the cones sampled). The current was transiently reduced by a brief flash of light. This is illustrated in Fig. 1a for a tiger salamander cone and Fig. 1b for a turtle cone. A bright light that elicits a saturating response turns off the dark current completely. The time course of the current response seen in salamander cones was very similar to the voltage response of salamander cones reported by other workers⁷, and was also similar in waveform to current responses in *Iguana* cones as measured with pairs of extracellular voltage electrodes⁸. However, current responses of turtle cones to bright flashes lacked the initial transient seen in corresponding voltage responses². We suggest that, as in toad rods³, the voltage transient seen in turtle cones is due to a voltage-sensitive conductance not located in the outer segment. The relationship between peak change in membrane current, I_p , and light intensity, L , was satisfactorily fitted by a Michaelis-Menten equation of the form $I_p/I_{\max} = (1 + S/L)^{-1}$, where $I_{\max}$ is the

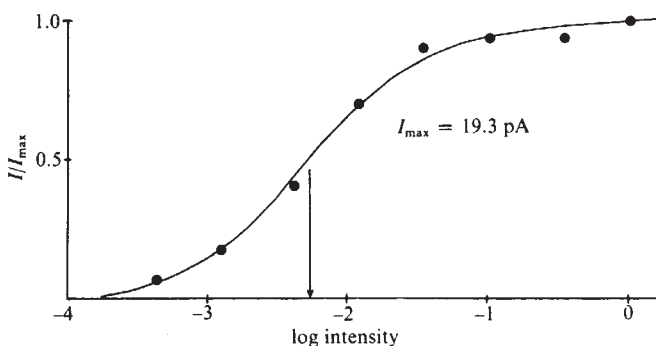


Fig. 2 Peak change in membrane current recorded from a turtle cone outer segment as a function of the intensity of a white, 10-ms test flash. For the cell shown in Fig. 1a. Intensity is displayed in log units relative to the maximum unattenuated stimulus. The calculated number of effective photoisomerizations for the unattenuated test light was 3.10×10^5 per flash. The value of the maximum current response, $I_{\max}$, was adjusted to compensate for signal attenuation due to current shunt through the sealing resistance between the electrode and the outer segment. Continuous curve is drawn from a template which described a Michaelis-Menten equation. The arrow, indicating the parameter S , is $10^{-2.26}$ of the unattenuated flash, corresponding to 1.70×10^3 effective photoisomerizations (S^*). From the Michaelis-Menten equation, the response to a single effective photon would be $19.3 (1 + 1.70 \times 10^3)^{-1} = 0.011$ pA. For narrow band spectral stimuli (width at half amplitude = ± 5 nm), the number of photons per μm^2 at all attenuation settings were measured by putting a photodiode (United detector 101A) in place of the retina. The number of effective photoisomerization per test flash, L^* , was estimated by the equation $L^* = 2.3\beta(\lambda)VLt$, where L is the light intensity in incident photons per μm^2 per s, t is the duration of the test flash, $\beta(\lambda)$ is the specific density of the cone pigment for unpolarized light of wavelength λ (refs 5, 6), V is the volume of the outer cone segment as calculated by measuring the outside dimensions of the cone with an eyepiece micrometer, and f is the fraction of absorbed photons that produced a change in membrane current. The fraction f , estimated as 0.5, was determined by substituting a toad retina for the turtle retina, counting the number of single current 'bumps' (L^*) elicited in a rod by dim light flashes, and dividing by the number of photons absorbed ($2.3\beta(\lambda)VLt$). To calculate photoisomerizations for a 'white' test flash, the intensity of a narrow band stimulus was adjusted so that the current response elicited by this test flash matched that to the white flash. L^* was then calculated from the above equation.

maximum change in membrane current and S is the number of incident photons required to produce a half-maximal change in membrane current, as shown in Fig. 2. In six experiments in turtle and tiger salamander, the average number of photoisomerizations needed to elicit a half-maximal response, S^* , was 2,150 (range 1,350–2,750). The method for converting light intensity in incident photons, L , to photoisomerizations, L^* , is given in Fig. 2 legend.

There was no detectable current response to dim flashes where approximately one photoisomerization was elicited per cone. This is not surprising because the substitution of $L^* = 1$ in the Michaelis-Menten equation $I_p/I_{\max} = (1 + S^*/L^*)^{-1}$, with $S^* = 2,150$, yielded an average value for the peak change in membrane current per photoisomerization of only 0.013 pA or 0.05% of the maximum current. Our recording system would have been unable to detect such a small change in current. Using a value of 30 mV (refs 2, 7, 9, 10) for the driving force on the membrane current in the dark, we calculate an average decrease in outer segment membrane conductance at the peak of a single photon response of 0.43 pS.

The fraction, B , of channels closed by a single photoisomerization can be determined from the equation $B = (1 + S^*\alpha)^{-1}$, where S^* is as before and α is the ratio of the membrane conductance of a cone during a saturating response and its membrane conductance in the dark². Taking our average value of 2,150 for S^* and an estimate for α of 0.67 (refs 2, 7), for dim flashes each effective photoisomerization blocks 0.07% of the available channels. This is about half the value obtained by Baylor *et al.*² based on voltage recordings of turtle cones. The difference may reflect a greater light-adapted state in this preparation. Assuming that this is the case, a single photoisomerization can be expected to elicit approximately a 10–12- μV change in membrane potential¹⁵, and taken with a 0.013-pA current event, this yields an input resistance of an isolated cone of about 800–900 M Ω . This value is close to the value estimated for the input resistance of isolated rods^{3,11}, but is larger than that previously estimated for turtle cones on the basis of their spatial sensitivity profiles¹².

In response to a step increase in illumination, we observed that the suppression of inward current reached a maximum and then dropped to a plateau in seconds, as shown in Fig. 3 for a light that transiently suppressed all the dark current. The time course of the decay depended on light intensity. Calculations based on those by other workers¹³ show that the decrease in quantum catching resulting from photopigment bleaching at these intensities has an insignificant role in the decay of the response to steps of light. On the basis of records obtained with the procedure illustrated in Fig. 3, we suggest that the most likely explanation for the decay in the current response is a reopening of outer segment ion channels that were initially closed during the peak of the response. At about 50 s after the onset of the light, when the current response had decayed and was maintained at a steady plateau level, the light intensity was increased. We observed a further reduction in membrane current. It seems that during the development of the plateau phase a population of unblocked channels had appeared and these were now available to be blocked by a process generated by the arrival of more photons. We suggest that these channels are derived from those blocked during the initial saturating phase of the response.

It has been reported for turtle cones that fluctuations in membrane potential around the mean value in the dark are substantially reduced when the cell is hyperpolarized by bright lights⁹. In weakly coupled cones, a decrease in the standard deviation, σ_v , of membrane voltage fluctuations of as much as 0.66 mV, or 5–6% of the maximum light response, has been observed. We are unable to support the suggestion that this noise is generated in the outer segment. If the voltage fluctuations were due to conductance fluctuations in the outer segment, the reduction in the standard deviation of the current elicited by light should also be 5–6% of the maximum light response, or about 2 pA. Although we have measured some changes in the fluctuations associated with records of outer-segment membrane current between darkness and various levels of illumination, we have not seen a reduction in current noise as large as that predicted from the records of voltage fluctuations of isolated cones. The largest reduction we observed in the standard deviation of the current going from darkness to a saturating light intensity was 0.37 pA in a 38-pA cone, or about 1% of the total current (after compensation for the current shunt through

the sealing resistance, bandwidth = d.c. – 10 Hz). As the state of adaptation of the two preparations seem comparable (judging from estimates of S^*), we conclude that a sizeable portion of the reported fluctuations in voltage is not generated in the outer segment.

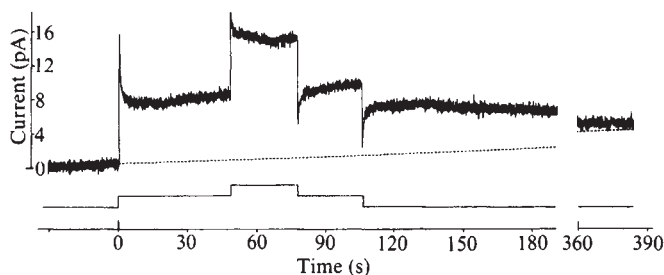


Fig. 3 The current responses of a turtle cone outer segment to steps of light (bandwidth, d.c. – 10 Hz). Uncorrected records representing an estimated 65% of the true current. Stimulus monitor shown below. Steps of illumination correspond to 6.80×10^4 followed by 2.18×10^5 photoisomerizations per s.

We cannot be sure that the current fluctuations we have observed are associated with the phototransduction process. The changes in variance are very small and not measurable in some recordings (especially recordings where the sealing resistance is low and consequently the baseline current noise is high). If the dark current noise that can be abolished in bright light is due to random fluctuations in the number of open ionic channels and these channels can be closed by light, the current i , flowing through each open channel, can be estimated from the equation $i = \sigma_i^2 / (\Delta I \cdot p)$, where σ_i^2 is the difference between the current variance in the dark and at the peak of a saturating response, ΔI is the mean change in current and p is the proportion of these channels that are already closed in the dark. Using our corrected values $\sigma_i^2 = 0.14 \text{ pA}^2$ and $\Delta I = 38 \text{ pA}$ from one turtle cone and taking $p = 0.3$ (ref. 14), we calculate a single channel current of 0.012 pA . This value is close to estimates of the size of the elementary current event underlying dark noise in rods^{1,14}. A comparison between this value and our estimate of the current change produced by a single photoisomerization in dim lights leads us to suggest that if this current noise is generated by fluctuations of light-sensitive channels in the outer segment, a single photoisomerization causes the closure of about one channel.

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Specific dephosphorylation of membrane proteins in Rous sarcoma virus-transformed chick embryo fibroblasts

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Chick embryo fibroblasts (CEF) infected with avian sarcoma virus become rapidly transformed as a result of expression of the viral *src* gene^{1,2} in the form of a single polypeptide of molecular weight 60,000 (pp60^{src})³ with protein kinase activity⁴ and suggested preferential association with the plasma membrane^{5,6}. Studies with normal avian and mammalian cells have revealed the presence of an antigenically related protein^{7,8} which seems to have similar kinase activity, but which is present at less than 1% of the levels of virally induced *src* protein found in transformed cells^{8,9}. As dynamic protein phosphorylation is important in numerous regulatory processes^{10,11}, the phenotypic expression of transformation may arise from an imbalance in one or more regulatory mechanisms that are controlled by protein phosphorylation^{4,8,12}. The cell membrane is affected during transformation¹³, including its phosphotransferase activity^{14,15}. The latter has been shown using isolated membrane fractions whose properties may be changed during preparation¹⁶. Therefore, we have compared the phosphorylation state of individual membrane proteins found in intact normal and RSV-transformed cells and report here the identification of two heavily phosphorylated, acidic membrane proteins in normal CEF which are specifically dephosphorylated on transformation by wild-type and temperature-sensitive Rous sarcoma viruses.

Normal and transformed chick fibroblasts were metabolically labelled with ³⁵S-methionine, and membranes were prepared by the method of Brunette and Till¹⁷ and analysed by two-dimensional gel electrophoresis. Two proteins of molecular weights 57,000 and 55,000 (57 K and 55 K proteins) and similar isoelectric point (~5.1) were identified as being almost exclusively associated with the membrane preparations (Fig. 1). That the membrane association was not an artefact of preparation was supported by the presence of these proteins in membranes prepared by sucrose density centrifugation (Fig. 1c, ref. 18). When normal cells (or cells infected with RAV-2, which does not contain the *src* gene) were incubated with ³²P-P_i, both proteins became heavily labelled within 2 h (Fig. 2a). In contrast, transformed cells labelled in identical conditions showed very little net incorporation of phosphate by these proteins (Fig. 2c). Quantification of both proteins by ³⁵S-methionine incorporation over 17 h suggested that any apparent net reduction in these proteins following cell transformation was insufficient to account for the dramatic decrease in phosphorylation. Normal cells and cells infected with RAV-2 virus showed no cell density dependence of phosphorylation of these proteins (data not shown).

We examined the turnover of phosphate on these proteins by means of a pulse-chase study (Fig. 2). Normal cells labelled with ³²P-P_i for 2 h (Fig. 2a) and chased for 4 h (Fig. 2b) showed a marked decrease in label associated with intermediate filament protein (IFP) and most other phosphoproteins, some increase in a 87 K protein, but no apparent decrease in the isotope associated with either the 57 K or 55 K proteins. This suggests that phosphatase activity specific for these phosphoproteins is limited in the normal cell or that these proteins are found in a privileged location that is inaccessible to general phosphatase activity. In contrast, little label is detected in these proteins at either time point in RSV-transformed cells (Fig. 2c, d). Other

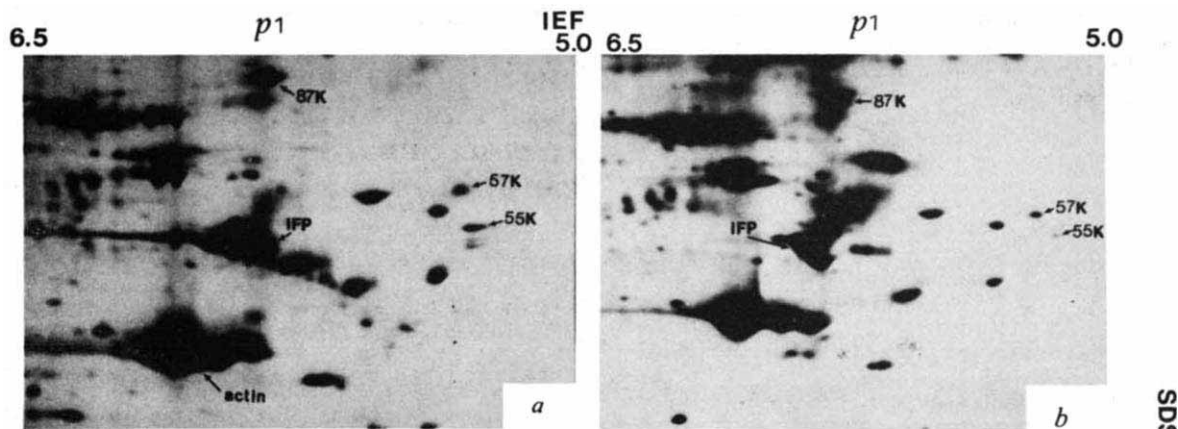


Fig. 1 Localization of 57 K and 55 K proteins in the plasma membrane. Uninfected and Rous sarcoma virus (Schmidt-Ruppin A strain)-infected CEF were labelled with ^{35}S -methionine for 18 h in Dulbecco's minimal essential medium (MEM) containing half the usual concentration of methionine. Membranes were then prepared by the method of Atkinson and Summers²⁰ and isolated by the technique of Brunette and Till¹⁷. The membrane proteins were analysed by two-dimensional gel electrophoresis as described by O'Farrell²¹ and modified by Ames and Nikaido²². The gels were dried on to filter paper and autoradiography was on Kodak X-Omat film. Only the relevant areas of the autoradiograms are shown. *a*, Autoradiograph pattern of normal membrane proteins; *b*, the corresponding pattern from transformed membranes; and *c*, plasma membrane vesicles prepared from normal cells by equilibrium centrifugation on a sucrose density gradient¹⁸, analysed in a similar fashion, and stained with Coomassie blue. It is apparent that there is some degree of association of structural proteins, that is, intermediate filament protein (IFP) and actin, with membranes, particularly those prepared by the Brunette and Till method¹⁷. The 57 K and 55 K proteins are invariably found to be membrane associated by both techniques. The presence of the 57 K and 55 K proteins could not be detected in the 100,000g supernatant from these preparations.

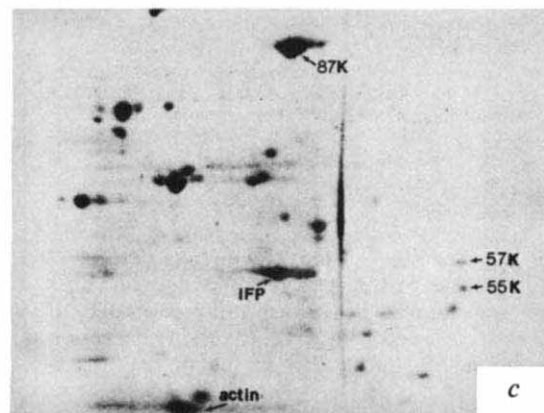
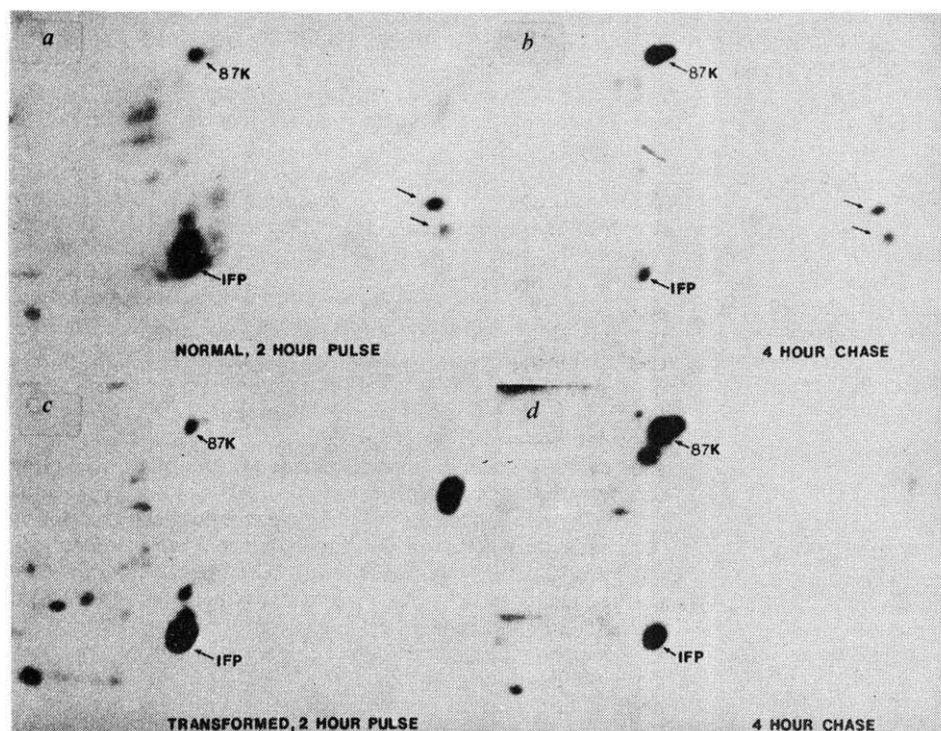


Fig. 2 Relatively slow turnover of phosphate on the 57 K and 55 K proteins. Normal chick fibroblasts (*a*) or Rous sarcoma virus (Prague C strain)-transformed fibroblasts (*c*) were incubated with 1 mCi ^{32}P -P_i in phosphate-free medium for 2 h. The cells were prepared directly for two-dimensional gel analysis by lysis in 2.3% SDS containing 5% mercaptoethanol, heating at 95 °C for 3 min, and adding 2 volumes of 'standard dilution buffer'²². Alternatively, following the 2-h pulse, the ^{32}P -labelled normal (*b*) or transformed (*d*) cultures were incubated for an additional 4 h in Dulbecco's MEM (Gibco H-21) with 5% calf serum and 10% tryptose phosphate broth. The samples were prepared as before and whole cell phosphoproteins analysed by two-dimensional gel electrophoresis. In this and following experiments, 10^6 acid-precipitable c.p.m. were loaded per gel. Autoradiography was for 24 h. The 2-h pulse resulted in significant labelling of the 57 K and 55 K proteins in normal cells (*a*, arrows). Although the 4-h chase resulted in loss of label from IFP and most other proteins except protein of molecular weight 87,000 in both normal and transformed cells, there was no significant decrease in label associated with these normal cell phosphoproteins (*b*, arrows) in four successive experiments.



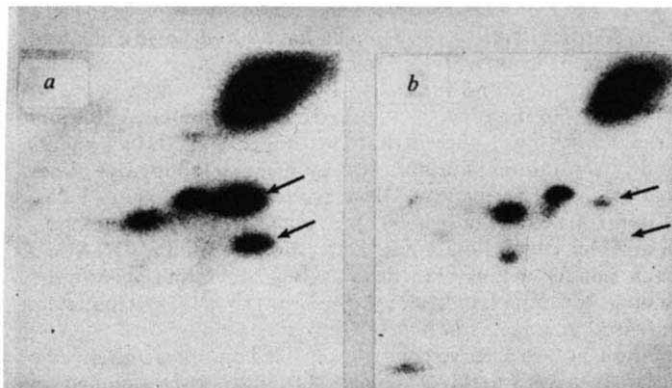


Fig. 3 Effect of growth temperature on phosphorylation of the 57 K and 55 K proteins in cells infected with the temperature-sensitive virus, tsNY68. Chick fibroblasts were infected with tsNY68 virus¹⁹ and incubated at 41 °C (a) or 35 °C (b). Examination of the cultures by phase microscopy revealed normal cell morphology at a growth temperature of 41 °C (non-permissive temperature) and transformed morphology at a growth temperature of 35 °C (permissive temperature). Cultures at both temperatures were incubated with 1 mCi ³²P-P_i per 60-mm plate for 2 h, then lysed in SDS and analysed by two-dimensional gel electrophoresis. Autoradiography was for 72 h. Labelling of the 57 K and 55 K phosphoproteins is seen to be heavy in cells grown at 41 °C, but is almost undetectable in cells grown at 35 °C. Temperature-shift experiments showed that detectable alterations in the phosphorylation state of these proteins occurred within 3–4 h of the temperature change. The two labelled spots seen to the left of the 57 K protein are normal cell phosphoproteins not seen in Figs 2 and 4 because of shorter exposure times.

phosphoproteins in the pulsed and chased transformed cells behaved in a similar fashion to that seen in normal cells.

It was important to determine whether dephosphorylation of the 57 K and 55 K proteins occurred in cells transformed by a temperature-sensitive mutant of Rous sarcoma virus (tsNY68). These cells manifest normal phenotype when maintained at a growth temperature of 41 °C, but show rapid and reversible morphological and biochemical transformation (within 6 h) when shifted to 35 °C (ref. 19).

We found a significant alteration in phosphorylation state of the 57 K and 55 K proteins when comparing cells grown at 41 °C (Fig. 3a) with cells grown at 35 °C (Fig. 3b). This phosphorylation state is temperature sensitive in both directions within 3–5 h of a shift in temperature, increasing when the temperature is raised and decreasing when the temperature is lowered (data not shown). Thus, it would seem that the dephosphorylation of these proteins is temporally linked to the onset of the transformed phenotype. The slow turnover of the phosphate on these proteins in normal cells (Fig. 2) when considered with these latter data, suggests the existence of a phosphatase activity in transformed cells that is specific for these phosphoproteins. Protein turnover as a significant factor in the dephosphorylation seems less likely, as ³⁵S-incorporation did not show a dramatic decrease in transformed cells and might simply result from some increased susceptibility of the dephosphorylated proteins to degradation.

We considered the possible existence of a specific phosphatase in a mixing experiment. Normal CEF or RSV-CEF were incubated with ³²P-P_i and disrupted by nitrogen cavitation. These homogenates were incubated, either mixed (Fig. 4c, d) or unmixed (Fig. 4a, b), at 41 °C for 4 h. The phosphoproteins were analysed by two-dimensional gel analysis and autoradiography.

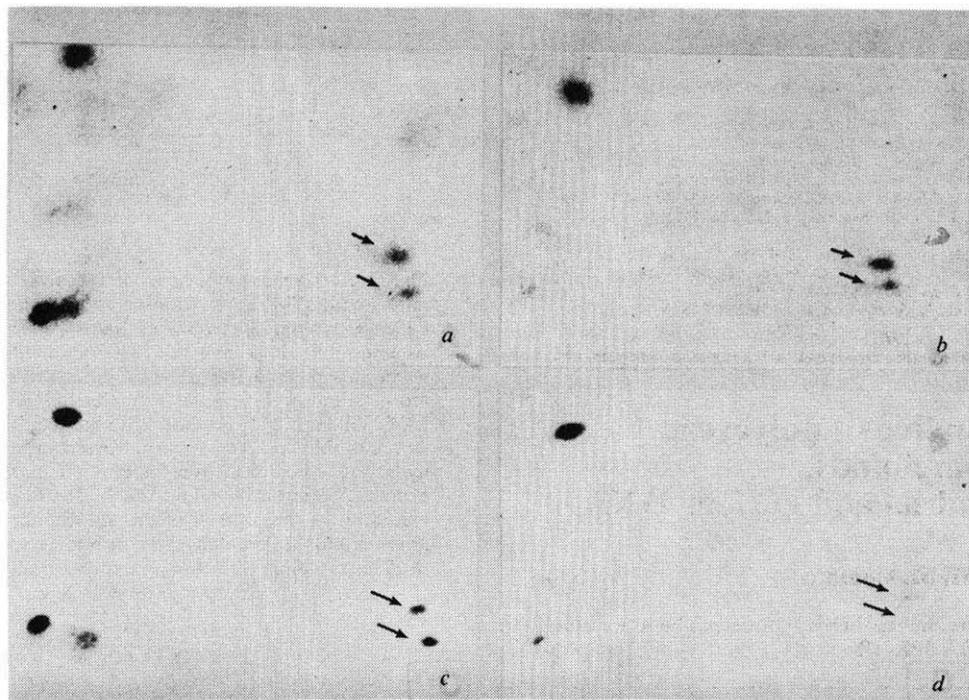


Fig. 4 Specific dephosphorylation of 57 K and 55 K phosphoproteins from normal cells by transformed CEF homogenate. Normal CEF were labelled with ³²P-P_i (1 mCi per 100-mm plate) for 2 h and disrupted by nitrogen cavitation (400 p.s.i. in a Yeda cell press) in Tris-Cl buffered saline, pH 8.0. Phase microscopic examination of the homogenate showed that fewer than 5% of the cells remained intact. Unlabelled normal cells were also disrupted in the same fashion and mixed with the homogenate derived from an equal number of the labelled cells. An aliquot of this suspension was prepared immediately for electrophoresis (a) while the remainder was incubated at 41 °C for 4 h at which time another aliquot was prepared for electrophoresis (b). No significant decrease is seen in the level of label in the 57 K and 55 K phosphoproteins although IFP and most other phosphoproteins show marked loss of isotope. The 87 K phosphoprotein is apparently stable to phosphate loss. RSV-transformed (Prague C strain) fibroblasts were disrupted by the same protocol and mixed with the homogenate from an equal number of disrupted ³²P-labelled normal CEF. This suspension of mixed homogenates was either prepared for electrophoresis immediately (c) or incubated at 41 °C for 4 h (d) before SDS solubilization. All samples were analysed by two-dimensional gel electrophoresis. Autoradiography was for 24 h. It is apparent that mixing of transformed cell homogenate with ³²P-labelled normal cell homogenate results in the specific dephosphorylation of the 57 K and 55 K phosphoproteins. Note that no decrease in label associated with the 87 K phosphoprotein is detectable in the mixed homogenates (c and d). These results were verified in three successive experiments.

Only in the mixed homogenate was specific decrease in the activity of these proteins seen (Fig. 4c, d). The remainder of the autoradiographic pattern obtained with the mixed homogenate was identical to that found with unmixed homogenates. The protein patterns as detected by staining with Coomassie blue remained unchanged in all cases during the 4-h incubation suggesting that general protease activity was not important in these changes.

Our results suggest the presence of a phosphatase activity in transformed cells which appears specific for the 57 K and 55 K phosphoproteins. The function of the 57 K and 55 K proteins is presently unknown although both are membrane associated. The nature of the putative specific phosphatase activity is uncertain although several possibilities come to mind. It is possible that the *src* protein may activate a phosphatase specific for the 57 K and 55 K phosphoproteins. On the other hand, the *src* protein itself may contain an as yet unidentified phosphatase activity.

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Covalent binding of polycyclic aromatic compounds to mitochondrial and nuclear DNA

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Since the pioneering work of the Millers¹ it has become clear that most chemical carcinogens require metabolism to reactive electrophiles and then exhibit their carcinogenic potential by reacting chemically with, and modifying, cellular macromolecules. At first modification of proteins was considered most likely to be of importance in carcinogenesis. Later, Brookes and Lawley² demonstrated that the extent of binding of several polycyclic hydrocarbons to DNA, but not to RNA or protein isolated from the skin of mice treated topically with these compounds, correlated with their known carcinogenic potency

to this tissue. Mammalian cells, particularly mouse embryo cells, treated with chemical carcinogens have often been used, and DNA has been involved almost exclusively from whole cells. However, mitochondria possess unique DNA which accounts for 0.1-1% of the total DNA present in mammalian cells³, and three studies have shown that carcinogenic alkylating agents modify the mitochondrial DNA by a factor about five times greater than the nuclear DNA from the same cells⁴⁻⁶. We demonstrate here that with six polycyclic aromatic compounds, all of which require metabolic activation and bind to DNA to a much smaller extent than direct-acting alkylating agents, the binding to mitochondrial relative to DNA is dramatically increased by a factor of nearly 50 to over 500.

Whole mouse embryo cells in their third passage from specific pathogen-free Tyler's Original (TO) mice were labelled in culture with [2-¹⁴C]thymidine (60 mCi mmol⁻¹) and grown to confluency. Tritium-labelled polycyclic compounds (0.16-1.25 µg ml⁻¹, depending on toxicity) were added as shown in Table 1 and 24-48 h later the cells were collected, washed, and

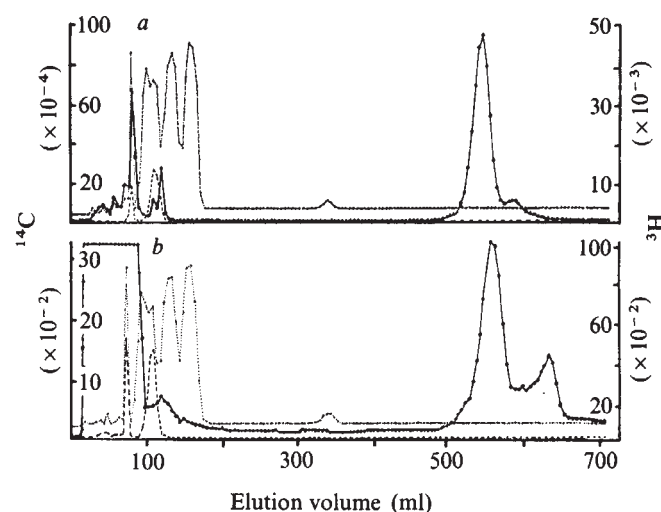


Fig. 1 LH20 chromatography of hydrolysates of nuclear (a) and mitochondrial (b) DNA from mouse cells treated in culture with benzo[a]pyrene. Hydrolysates were run on Sephadex LH20 columns of 90 × 1.5 cm with a linear gradient of 30% methanol in water increasing to 100% methanol. Fractions (5 ml) were collected and aliquots (1.5 ml) were counted for ¹⁴C and ³H activities in a LKB Wallac 1215 Rackbeta liquid scintillation counter. The amounts of the nucleosides were corrected for the efficiency of the hydrolysis, calculated from the total ¹⁴C in the DNA hydrolysed and the ¹⁴C in the thymidine peak. The large amount of tritium eluted in the first 100 ml of the mitochondrial DNA hydrolysate arises from the tritiated hydrocarbon bound to the RNA and residual protein. ·····, UV absorption at 254 nm; ●—●—●, ³H d.p.m.; ———, ¹⁴C d.p.m.

gently disrupted mechanically by hand using a Potter-Elvehjem homogenizer. Nuclei and mitochondria were isolated by the methods of Prescott *et al.*⁷ and Miyaki *et al.*⁸, respectively, and their purity was checked by light and electron microscopy. Nuclear DNA was extracted using Diamond's modification of the Kirby method⁹ which involves isolation of the DNA from SDS lysates by phenol extraction, subsequent treatment with RNase, and a second isolation by phenol extraction. Mitochondrial DNA was obtained basically by the method of Hirt¹⁰, because mitochondrial DNA was lost during phenol extraction, the latter step and RNase treatment were omitted. The resultant solution of mitochondrial DNA therefore still contained mitochondrial RNA and residual protein which were also labelled

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Table 1 Binding of polycyclic aromatic compounds to nuclear and mitochondrial DNA

Compound	Specific activity (Ci mmol ⁻¹)	Dose (µg ml ⁻¹)	Incubation time (h)	Binding index (µmol of compound bound per mol DNA phosphorous)		
				Nuclear DNA (nuc)	Mitochondrial DNA (mt)	Ratio mt/nuc
DMBA	19.6	0.212	24	12.1 ± 2.0	897 ± 63.5	78 ± 18.5*
MCA	9.0	0.496	24	21.9 ± 0.3	1,024 ± 20	47 ± 0.3*
B[a]P	18.0, 27.0	0.235	24	7.5 ± 1.9	413 ± 164	53 ± 8.7*
11-MeCPP	13.9	1.250	48	0.53	312	589†
B[a]A	5.9	0.644	24	0.44 ± 0.11	104 ± 40.2	230 ± 51.5*
12-MeCPP	27.5	1.250	48	0.28 ± 0.03	26.3 ± 16.2	98 ± 65.5*

All compounds were generally labelled with tritium. DMBA, 7,12-dimethylbenz[*a*]anthracene; MCA, 3-methylcholanthrene; B[a]P, benzo[*a*]pyrene; 11-MeCPP, 15,16-dihydro-11-methylcyclopenta[*a*]phenanthren-17-one²⁴; B[a]A, benz[*a*]anthracene; 12-MeCPP, 15,16-dihydro-12-methylcyclopenta[*a*]phenanthren-17-one²⁴.

* Duplicate experiments ± s.d.

† Single experiment.

with tritium through binding to the hydrocarbons to these macromolecules.

The amount of nuclear DNA (usually 1–2 mg) was determined by UV spectrophotometry, having calibrated this by phosphorous analysis using the colorimetric method of King and Wotton¹¹. Mitochondrial DNA was estimated, following enzymatic hydrolysis and separation of the deoxynucleosides, by relating the ¹⁴C d.p.m. in the thymidine peak to the specific ¹⁴C activity of the nuclear DNA, and was found to fall into the expected range (about 2 µg). On one occasion this was confirmed by direct estimation of the mitochondrial DNA by the fluorimetric micro method of Hill and Whatley¹² using mithramycin, thus establishing that the ¹⁴C specific activities of the nuclear and mitochondrial DNA were similar. After dilution with carrier calf thymus DNA, both samples were hydrolysed enzymatically with DNase which had been carefully checked for absence of RNase activity, and the liberated deoxyribonucleosides were separated by Sephadex LH20 chromatography (Fig. 1). The binding index (µmol of compound bound per mol of DNA phosphorous) was calculated from the total amount of tritium in the modified deoxyribonucleoside peaks which elute after the natural deoxyribonucleosides, assuming the specific activity of the bound compound was unaltered. As Phillips *et al.*¹³ have shown, residual protein and RNA do not interfere with this separation because they are eluted in the first 100 ml, immediately before the natural nucleosides. Six polycyclic aromatic compounds were examined in this way, the results of which are summarized in Table 1.

All six compounds modified both nuclear and mitochondrial DNA, and in each case the binding index for the latter was considerably higher than for the former. Although experimental difficulties made the precision of the values rather uncertain, the large differences observed cannot be explained by the small deviations in purine and pyrimidine base ratios known to exist between mammalian nuclear and mitochondrial DNA. These differences in binding index may occur because both the mitochondrial inner¹⁴ and outer¹⁵ membranes contain the enzymes required to metabolize polycyclic aromatic compounds which probably tend to accumulate in these lipophilic membranes¹⁶. Furthermore, mitochondrial DNA is 'naked', being devoid of the histone and non-histone proteins that are intimately associated with DNA in the nucleus. The highest DNA-binding indices, both nuclear and mitochondrial, are shown by the two most potent carcinogens: 7,12-dimethylbenz[*a*]anthracene (DMBA) and 3-methylcholanthrene (MCA). Benzo[*a*]pyrene and 15,16-dihydro-11-methylcyclopenta[*a*]phenanthren-17-one (11-MeCPP) are similar to each other in potencies¹⁷, but are less active as carcinogens than DMBA and MCA; benz[*a*]anthracene is an extremely weak carcinogen whereas 12-MeCPP is inactive¹⁸. The nuclear DNA-binding indices of these last four compounds do not reflect this, whereas there is good correspondence with the mitochondrial binding indices. In all cases, the nucleoside adducts seen in the nuclear DNA

hydrolysates are also present in the hydrolysates of the mitochondrial DNA, but in addition the latter also exhibit substantial amounts of slower-running adducts. In the case of benzo[*a*]pyrene¹⁹ (shown in Fig. 1) and 11-MeCPP²⁰, the major nuclear adduct peak is known to represent the compound formed by reaction of the bay-region dihydrodiol epoxide with deoxyguanosine. The slower-running, less polar adducts therefore probably result from reaction of simple non-hydroxylated epoxides, such as K-region or non-K-region epoxides, with a DNA base.

The significance of these findings is not clear. As these cells contain about 1,000 times more nuclear than mitochondrial DNA, the actual amount of compound bound to the former is 2–20 times that bound to the latter. This does not imply that damage caused by this modification will also be in this ratio, because the efficiency of DNA repair may be different at these two sites. However, mitochondrial DNA codes for several essential subunits of ATPase, cytochrome *bc*₁ and cytochrome *c* oxidase²¹, and it is therefore interesting that derangements of energy metabolism often characterize tumours (see ref. 22). Recently Wilkie *et al.*²³ found that most carcinogens they tested caused the cytoplasmic 'petite' mutation in the yeast *Saccharomyces cerevisiae*. Moreover, effects on the cell surface were observed in non-fermentable conditions where mitochondrial function is required for growth. These authors suggest a possible role for mitochondrial mutations in cancer through a heritable disturbance either directly or by modulation of a nuclear gene.

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Early capping of transcripts from the adenovirus major late transcription unit

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The various events which result in modification of a primary RNA transcript may have a role in choosing transcripts which are to be processed into mRNA. If there is a stepwise and interdependent nature to each of the modifications then it is important to place the various steps in temporal order. We show here that the formation of a capped 5' terminus seems to be a very early event for adenovirus type 2 (Ad-2) nuclear RNA that is initiated at the major late Ad-2 promoter¹⁻⁵. There is an equally rapid entry of ³H-adenosine into the cap and the first dozen or so adenylate residues in the RNA chain. Taken together with evidence on general mRNA metabolism in Chinese hamster cells⁶, it seems unlikely that capping has any differential role in successful RNA processing but rather is an automatic event for all RNA polymerase II products.

Location of the apparent initiation site for the major late adenovirus transcription unit near map position 16 on the genome (refs 3-5); 1 unit = 360 bases; 0 is at the 3' end of the rightward-transcribed strand) followed by the determination of DNA base sequence in this region⁷⁻⁹ showed that the common late 5' mRNA sequence m⁷GpppACUCUUCUG occurs only once near position 16 (refs 7-9). It therefore seemed likely that the initiating nucleotide in the primary transcript is the nucleotide which becomes capped during RNA processing. The viral DNA between 16.45 and 18.2 map units contains 536 bases of which 94 are thymidylate residues that are transcribed into adenylate residues in the primary RNA transcript as transcription proceeds from position 16.45 to position 18.2 (ref. 7). Together with the sequence information, an assay of chain length and a measurement of ³H-adenosine in the labelled chains and in the cap, the frequency of capping as a function of chain size can be estimated. For instance, an RNA chain 32 nucleotides long would contain four AMP residues one of which would be in the cap; therefore, 25% of the radioactivity in such a molecule labelled with ³H-adenosine would be in the cap.

Late in adenovirus infection, cells were labelled with ³H-adenosine for 20 min and the total nuclear RNA that sedimented more slowly than 18S was hybridized to SmaF DNA (11.6-18.2 map units) that was immobilized on nitrocellulose filters. The hybridized RNA remaining after T1 RNase digestion was then eluted from the DNA. In the first experiment (Fig. 1) the presence of caps in the SmaF-specific RNA was determined by digesting the RNA to completion with RNases A and T2 and P₁ nuclease followed by treatment with bacterial alkaline phosphatase to release labelled adenosine residues from internal positions in the RNA chain and labelled nuclease-resistant 'cap core' structures^{1,6}. Guanosine residues should also be labelled to the extent that ³H-adenosine is converted to guanylate nucleotides. After electrophoresis of the digest (pH 3.5) the majority of radioactivity was represented by adenosine (Fig. 1), with also a small amount of guanosine. In addition, there was a peak of labelled, negatively charged material (fractions 38, 39, Fig. 1) that was resistant to the nuclease digestion and that migrated similarly to the appropriate cap core marker (m⁷GpppAm).

About 2.7% of the total ³H-adenosine radioactivity was in the form of the presumed cap structures (Table 1), suggesting a chain length of about 175 nucleotides if every chain were capped. As the size of the short promoter-proximal chains that were analysed was about 200 nucleotides (see Fig. 2a), it seemed that most of the chains must be capped. Because at least

some of these molecules represent prematurely terminated transcripts^{10,11}, these results would indicate that the presence of a cap on an RNA transcript is not sufficient to ensure the completion of the chain.

It remained possible that some of the smallest promoter-proximal chains might be uncapped and contain pppA termini. Therefore the small RNA chains less than 18S were refractionated by sedimentation for a longer time through a second sucrose gradient (Fig. 2a). The peak of SmaF-specific RNA was in chains about 100-200 nucleotides long with significant hybridizable RNA both larger and smaller than tRNA (80 nucleotides). A 'large pool' from this gradient (fractions 2-7) represented chains longer than about 100 nucleotides, and a 'small pool' (fractions 8-12) contained molecules less than 100 nucleotides long. The promoter-proximal RNA was selected from both the large and small RNA pools by hybridization to the SmaF fragment. To detect potential uncapped termini that retained terminal phosphates, the nucleotide analyses were performed differently from those in the first experiment. After selection by hybridization, the RNA was digested only with P₁ nuclease and not with bacterial alkaline phosphatase so as to liberate labelled internal A or G residues as 5' nucleotides and leave 5'-terminal adenylate residues intact as either cap structures or uncapped polyphosphate residues^{1,6}. After DEAE-paper electrophoresis (pH 3.5), both large and small RNA samples yielded pA and pG residues along with material migrating similarly to the cap core marker and the ADP marker (peak C, Fig. 2b, c). In addition there were two minor peaks (A and B, Fig. 2b, c) of P₁-resistant material that were completely resistant to further digestion by alkaline phosphatase and therefore did not contain terminal phosphates (data not shown).

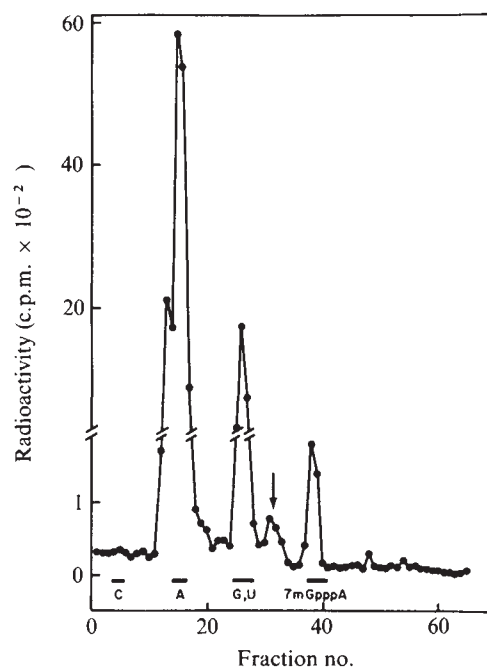


Fig. 1 Analysis of late Ad-2 promoter-proximal RNA for cap content. Adenovirus-infected cells at 16 h post-infection were labelled for 20 min with ³H-adenosine (300 μ Ci ml⁻¹). The nuclear RNA was prepared and molecules of less than 2,000 nucleotides were isolated by sucrose gradient sedimentation. The promoter-proximal RNA was selected by hybridization to filter-bound SmaF DNA (map coordinate 11.6-18.2). After removal of unhybridized RNA and tails by digestion with T1 RNase, the filter was washed with 2 $\times$ SSC and then incubated with iodoacetate to inactivate the RNase. The RNA was eluted in H₂O by heating to 100 $^{\circ}$ C for 2 min. The RNA was first digested with RNase A (200 units ml⁻¹) and T2 (20 units ml⁻¹), followed by digestion with P₁ nuclease (200 units ml⁻¹) and then bacterial alkaline phosphatase (20 units ml⁻¹). The digest was analysed by electrophoresis on Whatman 3MM paper at pH 3.5. The arrow indicates the origin. The positive electrode is to the right.

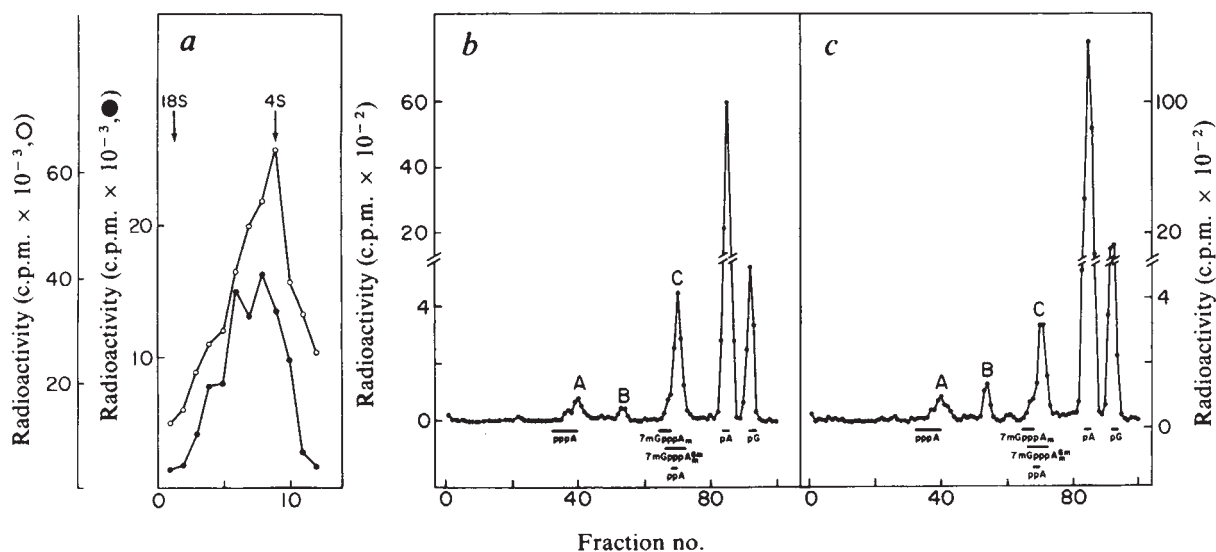


Fig. 2 *a*, Sedimentation profile of small, promoter-proximal late adenovirus nuclear RNA. RNA prepared from infected cells as described in the legend to Fig. 1 was resedimented in a second sucrose gradient (15–30% sucrose, SW41 rotor) at 30,000 r.p.m. for 19 h at 22 °C. Fractions of 1 ml were collected. Aliquots (10 μ l) of each fraction was counted directly in a Triton–toluene scintillant (○) and 50 μ l of each fraction was assayed by hybridization to nitrocellulose filters containing the *Sma*F fragment (●). Two pools were made for subsequent analysis. A pool representing large RNA included fractions 2–7; a small RNA pool included fractions 8–12. Analysis of large (*b*) and small (*c*) promoter-proximal RNAs for 5' termini. The two pools of RNA prepared as described in the legend to Fig. 2 were ethanol-precipitated and then each RNA sample was hybridized to filters containing the *Sma*F fragment. The eluted RNA was digested with P_1 nuclease (200 units ml^{-1}) and then electrophoresed on DEAE-paper at pH 3.5. Half of each lane was cut into 0.5-cm wide strips and counted. The other half was eluted and further analysed as described in Fig. 3 legend. The origin is at fraction 0 with electrophoresis towards the positive electrode.

Rather, this material could represent either ring-opened cap structures¹² or an incomplete P_1 digestion product. The major P_1 -resistant material (peak C, Fig. 2*b, c*), which constituted 70–80% of the non-pA radioactivity, migrated during electrophoresis on DEAE paper as either a cap core ($m^7GpppAm$) or ADP. Treatment of this peak with alkaline phosphatase alone showed that the peak C material from both the large and small pools was resistant to alkaline phosphatase digestion and migrated with the cap core marker (Fig. 3*b, d*). The material from peak C was, however, sensitive to a combined pyrophosphatase and alkaline phosphatase digestion yielding labelled adenosine (Fig. 3*a, c*), confirming that it was in fact cap core. Thus there was no evidence for terminal phosphorylated structures even in the smallest RNA chains.

Again, in this experiment, the proportion of the radioactivity in caps compared with that in adenylate residues in the RNA chains indicated that all chains were capped. There was a ratio of one A in the cap per 30 As in the chain (or about 160 total residues) for RNA molecules from the larger pool and 1 in 12 (or about 70 total residues) for the smaller pool (Table 1). These estimates of chain length of capped molecules are in good agreement with the sedimentation sizes of the two RNA pools, indicating that nearly every chain possesses a cap, including those transcripts which average only 70 nucleotides and therefore must include many chains considerably shorter than 70 bases. Furthermore, no evidence of uncapped phosphorylated termini could be found even in the short chains. It should be pointed out that free pppA and pppG termini are readily detected in short cellular RNA transcripts that are presumably polymerase I and polymerase III products⁶.

A previous analysis of the prematurely terminated transcripts from the major late adenovirus transcription unit demonstrated that in steady-state labelling conditions a prominent species of promoter-proximal RNA of chain length 200–250 nucleotides was detected¹¹. Very few prematurely terminated RNA chains in the size range of 80–200 nucleotides were observed. This is not the situation for the promoter-proximal RNA prepared from cells labelled for 20 min with 3H -adenosine where there are many chains in the size range of 100–200 nucleotides. Thus, it is likely that the chains which are less than 100 nucleotides long represent to some considerable degree, growing chains.

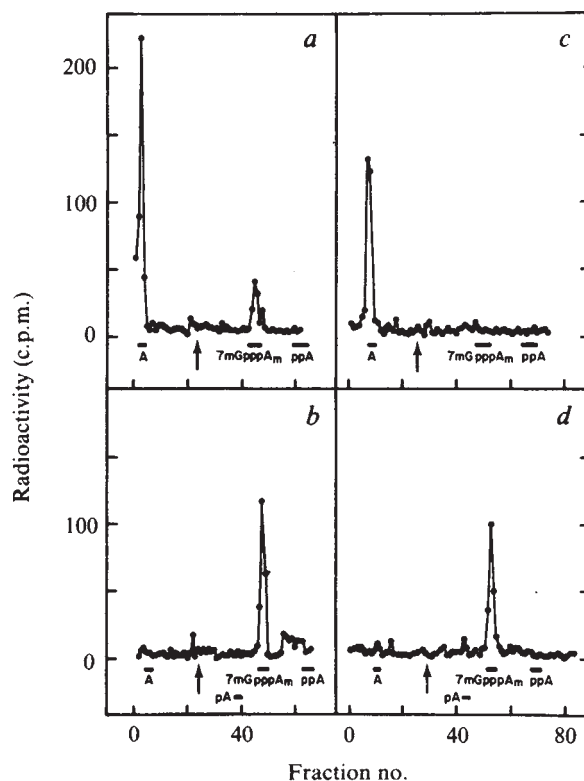


Fig. 3 Analysis of 5'-terminal structures from large and small promoter-proximal RNAs. The material designated as peak C in Fig. 2*b* and *c* was eluted from the DEAE paper in 30% triethylammonium carbonate, pH 7.4. The eluted material was lyophilized, dissolved in H_2O and then lyophilized again. The large RNA (*a, b*) and the small RNA (*c, d*) samples were divided into two parts. One part of each sample was digested with alkaline phosphatase alone. The other part was first digested with pyrophosphatase (1.5 units ml^{-1}) and then with alkaline phosphatase. The digestion products of each sample were then separated by electrophoresis on Whatman 3MM paper at pH 3.5. Strips 0.5 cm wide were counted. *a, c*, Pyrophosphatase plus alkaline phosphatase. *b, d*, Alkaline phosphatase alone. The arrow indicates the origin.

Table 1 Distribution of ^3H -adenosine label added late in adenovirus infection

Expt	RNA pool	Radioactivity (c.p.m.)			% Radio-activity as cap	Predicted chain length
		As adenosine	As pA	As cap		
1	—	16,801	—	468	2.7	175
2	Large	—	27,591	978	3.4	153
	Small	—	11,061	1,085	8.9	70

The values for expt 1 are from the data presented in Fig. 1, and for expt 2 from the data presented in Fig. 2b, c. The predicted chain lengths are based on the location of thymidylate residues in the sequence of Ad-2 DNA between the start site for late transcription at 16.45 map units and the *Sma*I cleavage site at 18.2 map units⁷. For each RNA chain, the cap produced by RNase A and T2 digestion would contain one A residue.

Since these short chains had the highest cap content and did not reveal any pppA termini it is clear that capping occurs soon after synthesis is initiated. Our experiments cannot determine whether the 5' terminus is capped concomitantly with the initiation of synthesis, although this is a possibility. Furuichi¹³ has demonstrated that the formation of a capped 5' terminus on the cytoplasmic polyhedrosis virus mRNA is a pre-transcriptional event. Weil *et al.*¹⁴ also showed that correct initiation of Ad-2 transcription can be obtained *in vitro* by RNA polymerase II in the presence of a crude cell extract. This crude *in vitro* system produces chains with capped ends. In addition to Ad-2 transcripts, polymerase II cellular transcripts also have caps added early during synthesis⁶. Thus an association of capping enzymes with the initiation complexes for polymerase II transcription is a distinct possibility.

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The Archaeobacteria and eukaryotic origins

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Critical analysis of the phylogeny of prokaryotes is in its infancy. Woese and others¹⁻⁵ have made the startling proposal that methane-producing bacteria and a few others form a phyletically unified group, the Archaeobacteria, as old and as diverse (although not now as numerous) as all other bacteria. The only critique⁶ of this proposal is inadequate⁷. Here we present an alternative view, that the Archaeobacteria were derived from other bacteria and contain the ancestor of a cell which engulfed others, eventually to become the first eukaryote.

The main evidence used for the relative time of divergence of the Archaeobacteria is that their 16S ribosomal RNA is very different from that of other bacteria. However, as Woese recognizes³, this can be the result of rapid early evolution. The origin of new adaptations often occurs rapidly, as exemplified by the evolution of bats⁸, whales⁹ and modern placental mammals as a whole¹⁰. Evolutionary rates vary widely¹¹, and an attempt¹² to extrapolate the average rate of evolution of cytochrome *c* from vertebrates to angiosperms gave results which contradicted the fossil record¹³. (We do not mean to dispute the usual approximate constancy of protein evolution.) Proteins themselves often evolve unusually rapidly when they undergo a major change in function^{14,15}. Even the supposedly nearly invariant histone H4 is very different between ciliates and other eukaryotes¹⁶. The suggestion⁵ that the diversity of Archaeobacteria argues against rapid evolution near their origin is a non sequitur; placental mammals are a familiar counter-example in which both phenomena occur. The only studied genomes of archaeobacteria are small^{17,18}, although far from the smallest known for prokaryotes¹⁹.

Our proposal (Fig. 1), based in part on work by Margulis²⁰, Hall²¹ and others, takes the evolution of mechanisms for acquisition of free energy to be central. We discuss the general topic elsewhere²². Any phylogeny must be based on a clear recognition of primitive states and derived states, for shared primitive states give no phyletic information^{23,24}. A set of differences is not in itself an adequate basis for a phylogeny, in part because the base of the phylogeny can then be anywhere. We take glycolysis alone (as in some lactic-acid bacteria), especially its later stages, to be primitive among surviving organisms because it is almost universally distributed and provides materials for other, presumably derived, processes such as the completed tricarboxylic-acid cycle.

The Archaeobacteria are moderately derived prokaryotes in this view, although most surviving prokaryotes are even more derived. Why then should they be so divergent from the others? The answer may be lost in the Archaean, but we can offer an initial suggestion. One of the free-living Archaeobacteria, *Thermoplasma*, lacks a cell wall, while the other known Archaeobacteria have a wide diversity of cell walls²⁵ all very different in composition from the walls of all other walled cells. Thus it seems as though the ancestral lineage of Archaeobacteria lost its cell wall, possibly for flexibility in locomotion or for energy conservation. Such a change could be related to other changes in adaptation.

Moreover, *Thermoplasma* itself shows several specific and derived similarities with eukaryotes²⁶. It has a histone-like protein which produces nucleosome-like condensations of DNA. It has actin- and myosin-like proteins and microbody-like respiration. It can invaginate its plasma membrane, although apparently not with fully developed endocytosis. Such an ability is necessary for a cell which engulfs others.

However, like other surviving Archaeobacteria²⁷, *Thermoplasma* has lipids with branched chains and with ether linkages instead of the esterifications of other prokaryotes and eukaryotes²⁸. Unless eukaryote lipids came from another member of the presumptive symbiosis, this suggests that the surviving Archaeobacteria evolved their unique lipids and other traits after the ancestral eukaryote diverged.

Halobacterium, a surviving archaeobacterium²⁹ with independently derived light-driven ATP synthesis^{18,30}, has other specific and derived similarities with eukaryotes. Its light-transducing pigment is almost identical to rhodopsin¹⁸. Its initiator tRNA resembles that of eukaryotes in some ways¹⁸ and produces a non-formylated methionine¹⁸, as in eukaryotes. The only ribosomal protein studied has a partial amino acid sequence nearly intermediate between those of the bacterium *Escherichia* and the yeast *Saccharomyces*¹⁸. *Halobacterium* has a ferredoxin rather similar to that of plants¹⁸, and its cell wall is composed of a glycoprotein resembling those of eukaryotic cell walls¹⁸. It even seems to have more than one non-plasmid chromosome, perhaps several¹⁸. Another archaeobacterium,

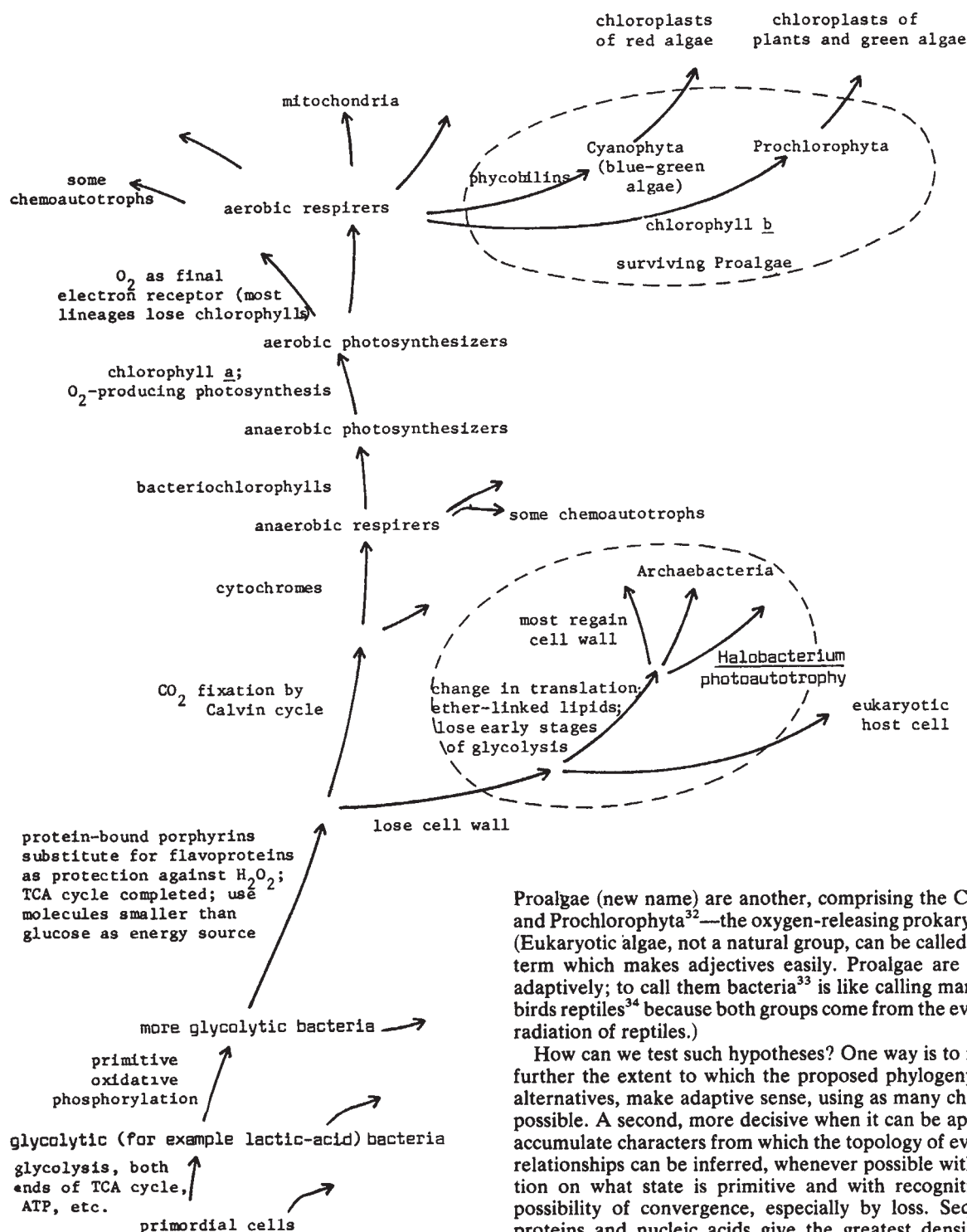


Fig. 1 Provisional phylogeny of prokaryotes.

*Methanobacterium*¹⁷, but not *Halobacterium*¹⁸, may have some repeated DNA.

Perhaps those resemblances to eukaryotes (except probably the cell wall) and those of *Thermoplasma* were present together in an ancestral form (*Thermoplasma* has not been studied for all of these characters) and some have been modified in each lineage. All enzymes of the tricarboxylic-acid cycle occur in archaebacteria, but not all together in the same species⁵. Parasitic mycoplasmas also show some specific resemblances to *Thermoplasma*^{25,31}; their relation to Archaebacteria needs clarification.

We therefore provisionally regard the Archaeobacteria as one of several phyla of prokaryotes, most as yet undefined. The

Proalgae (new name) are another, comprising the Cyanophyta and Prochlorophyta³²—the oxygen-releasing prokaryotic algae. (Eukaryotic algae, not a natural group, can be called eualgae, a term which makes adjectives easily. Proalgae are true algae adaptively; to call them bacteria³³ is like calling mammals and birds reptiles³⁴ because both groups come from the evolutionary radiation of reptiles.)

How can we test such hypotheses? One way is to investigate further the extent to which the proposed phylogeny, and any alternatives, make adaptive sense, using as many characters as possible. A second, more decisive when it can be applied, is to accumulate characters from which the topology of evolutionary relationships can be inferred, whenever possible with information on what state is primitive and with recognition of the possibility of convergence, especially by loss. Sequences of proteins and nucleic acids give the greatest density of such characters, but the data on rRNA (not sequences because only isolated oligonucleotides have been studied) suggest that so great changes may have occurred in the origins of both archaebacteria and eukaryotes that even such slowly evolving molecules show only random similarity to those of their ancestors. Primitive eukaryotes such as *Eimeria*³⁵ and *Pelomyxa*³⁶ also deserve intensive scrutiny.

The translation apparatus of Archaeobacteria differs from that of other organisms⁴. Perhaps there is an unbridgeable adaptive valley in this difference³. However, we think that this matter cannot be discussed adequately until much more is known about the functioning of existing translation apparatuses, and of potential intermediates, in realistic conditions. Until then we prefer to emphasize other evidence; it is dangerous to claim something is impossible in biology. The same function can be performed very differently in different selective conditions³⁷.

Nevertheless, Fig. 1 is consistent with existing evidence from macromolecular sequences³⁸ if primitive aerobic photosynthesizers are now extinct. Reticulate evolution by gene transfer provides an available and plausible excuse for disparities such as the presence in *Halobacterium* of a normal complement of cytochromes in an electron-transport chain³⁰, but there is a surprising lack of compelling evidence for its importance in any real case.

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Archaeobacterial elongation factor is ADP-ribosylated by diphtheria toxin

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Archaeobacteria have been defined as a 'third primary kingdom' of cells in addition to the eukaryotes and the eubacteria¹. While the latter two correspond approximately to the conventional categories eukaryotes and prokaryotes respectively, the Archaeobacteria have up to now comprised four groups of microorganisms: the methanogenic bacteria, the extremely halophilic bacteria and the two thermoacidophilic genera *Sulfolobus* and *Thermoplasma*. Based on ribosomal RNA sequence homologies and lipid composition, they apparently form a distinct group. Furthermore they possess or lack typical biochemical markers of both the eukaryotes and the prokaryotes, as well as having unique properties not found elsewhere². Altogether, this indicates that they are not closer to either one of the classical categories. One clear-cut difference between prokaryotes and eukaryotes is the diphtheria toxin reaction, which catalyses the covalent binding of adenosine diphosphate–

ribose (ADPR) to the eukaryotic peptide elongation factor EF2 in contrast to the homologous prokaryotic factor EF-G^{3,4}. We report here that diphtheria toxin also catalyses the ADP-ribosylation of archaeobacterial elongation factors. In this respect, these factors have to be assigned to the EF2 type; we suppose that the ADP-ribosylatable structure arising so early in evolution is of fundamental importance for the elongation process.

We have investigated crude cytoplasmic fractions of four main representatives of the Archaeobacteria: *Halobacterium cutirubrum*, *Methanobacterium thermoautotrophicum*, *Sulfolobus acidocaldarius* and *Thermoplasma acidophilum*. After reaction with diphtheria toxin fragment A and ¹⁴C-NAD labelled in the adenine moiety only one protein received a label. This could be demonstrated as radioactivity precipitable by trichloroacetic acid (TCA) and as one radioactive band in SDS-gel electrophoresis (Fig. 1). While *M. thermoautotrophicum*, *S. acidocaldarius* and *T. acidophilum* required the normal conditions³ for the toxin reaction (neutral pH, about 0.05 M K⁺, 30 min, 37 °C), high salt (3.8 M K⁺ or 2.6 M NH₄⁺) and 4 h incubation time were necessary for the extremely halophilic extract to attain maximum labelling. This reflects that the halophilic protein could only react in its native, salt-stabilized conformation, while the toxin fragment A, which has been shown to be remarkably stable to heat⁶, extreme pH⁶ and proteolytic enzymes⁴, also tolerates high salt.

For comparison, a number of elongation factors from various eukaryotic sources were ADP-ribosylated in the same manner as the archaeobacterial proteins; they showed the same mobility in one electrophoresis system, indicating a molecular weight (*M_r*) of 96,000. Isotopically labelled proteins from *M. thermoautotrophicum*, *S. acidocaldarius* and *T. acidophilum* were considerably smaller with apparent *M_r* of 86,000 for *M. thermoautotrophicum* and 83,000 for both the thermoacidophiles. These values are in the range of the *M_r* reported for the prokaryotic, non-ADP-ribosylatable homologue EF-G, which is about 80,000 (ref. 7). The *M_r* of 111,000 found for the halophilic protein is exceptionally high. The EF-Gs from *Escherichia coli*⁸ and *Streptococcus faecalis*⁹ as well as mitochondrial factors¹⁰ were shown to be unable to react with NAD and diphtheria toxin. We confirmed this for *Pseudomonas maltophilia*, *Bacillus licheniformis*, *E. coli* K 12, *Hyphomicrobium 526* and *Pedomicrobium* S 122.

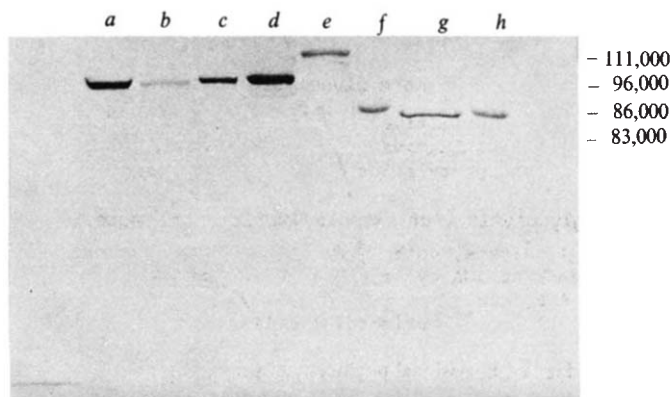


Fig. 1 Products of diphtheria toxin reaction from eukaryotic and archaeobacterial sources. Crude fractions of soluble proteins were incubated with mercaptoethanol-reduced toxin (10–20 flocculation units per 75 μ l), ¹⁴C-NAD (10⁻⁶ M) and 0.05 M KCl for 30 min at 37 °C. Different conditions were used for the extreme halophile: saturated potassium chloride and incubation time of 4 hours. The reaction mixtures were separated on polyacrylamide gels (7.5%)⁵, which were autoradiographed for 6 days. Calibration proteins were phosphorylase b (*M_r* 94,000), bovine serum albumin (*M_r* 67,000), catalase (*M_r* 58,000) and ovalbumin (*M_r* 43,000). The sources were as follows: a, wheat germ; b, rabbit liver; c, rat thymocytes; d, bovine liver; e, *H. cutirubrum*; f, *M. thermoautotrophicum*; g, *S. acidocaldarius*; h, *T. acidophilum*. Numbers on the right indicate *M_r*.

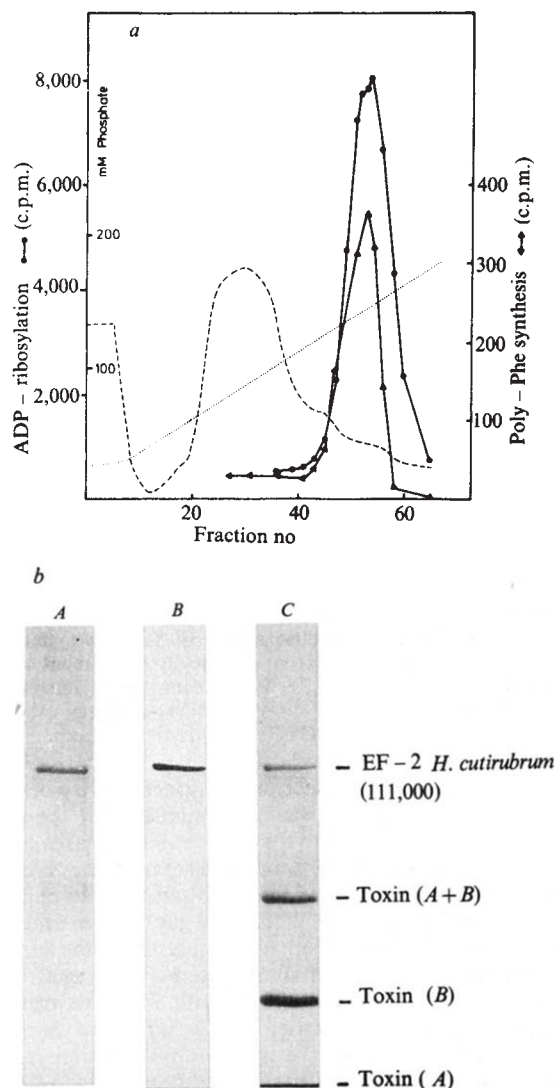


Fig. 2 *a*, Co-chromatography of an elongation factor and ADP-ribosylatable protein from *H. cutirubrum* on hydroxyapatite. Purified elongation factor was eluted by a linear gradient raising from 30 to 300 mM phosphate (pH 7.6), keeping the potassium concentration constant at 3.1 M by KCl. A_{280} is indicated as a dashed line in arbitrary units. Elongation factor activity (▲) was measured as poly-Phe synthesis in a cell-free system at saturated KCl as described¹², after removing inhibiting phosphate from the fractions by dialysis against 2.2 M $(\text{NH}_4)_2\text{SO}_4$ -buffer. A blank was subtracted consisting of ribosomes plus EF-1 equivalent. 55- μl aliquots of the fractions (2.2 ml) were tested in ADP-ribosylation assays (●) as in Fig. 1; bovine serum albumin was added to a concentration of 5.7 mg ml⁻¹, the mixture precipitated by TCA and filtered through glass fibre filters. *b*, SDS-electrophoresis⁵ of purified elongation factor. Lane A, Coomassie blue stain; lane B, autoradiography of purified, ADP-ribosylated factor; lane C, Coomassie blue stain of lane B, containing the toxin bands.

Further investigation of the extremely halophilic archaeobacterium *H. cutirubrum* identified the labelled protein with an elongation factor. For the protein synthesis test we modified a test system developed by Bayley¹¹, using poly(U) as a messenger, tRNA Phe as substrate and an EF-1-equivalent from *H. cutirubrum*¹², which complements with EF-2 to poly-Phe synthesis. The EF-2 co-purified with the ADP-ribosylatable protein using a three-step procedure on Sepharose with a decreasing $(\text{NH}_4)_2\text{SO}_4$ gradient, gel filtration at 0.9 M KCl/1.1 M $(\text{NH}_4)_2\text{SO}_4$ and hydroxyapatite (details to be reported elsewhere). The factor elutes from hydroxyapatite at 150 mM phosphate and is nearly homogeneous (Fig. 2*a*). The purified factor reacts with NAD and toxin as described for the crude fraction at high salt conditions. Under modified

purification procedures we have also observed a main Coomassie blue band in SDS electrophoresis of M_r about 93,000 (ref. 12), probably due to proteolysis of the factor. The active, ADP-ribosylatable elongation factor from *H. cutirubrum* has a M_r of 111,000 in SDS-electrophoresis (Fig. 2*b*).

The structural basis of the diphtheria toxin reaction in eukaryotic systems is well established: the ADP-ribose residue of NAD is bound covalently to a post-translationally modified histidine¹³ (previously called 'X', ref. 14), which only occurs in EF-2. The amino acid sequence of the ADP-ribosylation site has been shown to be highly conserved in yeast, wheat germ, rat and bovine liver¹⁵. It is obvious that the Archaeobacteria also provide the prerequisites for the toxin reaction. Taking into account the high specificity of the toxin for EF-2 and also the evidence from the halophilic system, we conclude that the ADP-ribosylated protein in archaeobacteria is an EF-2-type elongation factor. The eukaryotic feature of this elongation factor is a good argument for the separation of Archaeobacteria from the prokaryotes as a 'third primary kingdom' (ref. 1). The evolutionary pressure also seems to have worked over the large phylogenetic distance from Archaeobacteria to higher vertebrates. This consideration is further strengthened by results about ribosomal proteins, which are probably part of the binding site for the elongation factors on the ribosome¹⁶. These ribosomal A-proteins (L7/L12-equivalents) show a high degree of N-terminal sequence homology between halobacterial and eukaryotic systems¹⁷. Thus conservation of these cooperating structures may indicate functional importance for the elongation process in urkaryotes and their possible ancestors.

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Biophotolytic H_2 production using alginate-immobilized chloroplasts, enzymes and synthetic catalysts

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Hydrogen can be produced by illumination of an aqueous mixture of chloroplasts and hydrogenase, in the presence of an electron carrier^{1,2}. This system may have potential for development of a solar converter to produce hydrogen from water^{3,4} if it can be stabilized or constructed as a completely synthetic system. The immobilization of the chloroplasts, or membrane analogues, would make possible a one-stage reactor with all the components in one chamber, or a two-stage reactor if the electron carrier was passed to another chamber to react with

an immobilized hydrogen-producing catalyst. However, techniques for immobilizing enzymes tend to yield immobilized chloroplasts that are not very active, and other methods must be used⁵⁻⁸. We describe here the immobilization of chloroplasts using calcium alginate gels on reinforcing grids of nylon and stainless steel. Chloroplasts thus immobilized are fully active and can be used to produce hydrogen gas. Strengthened films of this sort could provide a good, solid, rigid matrix for a solar converter.

Sodium alginate is a soluble polysaccharide, but calcium alginate is an insoluble gel. If a chloroplast suspension is mixed with a 1 or 2% sodium alginate solution and then brought into contact with 0.1 M calcium chloride, the calcium alginate gel forms immediately. Thus, if the mixture is dropped into calcium chloride from a syringe, the drops solidify on contact and form nearly spherical balls, with the chloroplasts immobilized inside. This approach was used successfully for the immobilization of microbial cells by Cheetham *et al.*⁹. The same method was used in our laboratory and the spheres were found to be active in the hydrogen production system⁸ though they tended to disrupt after some time. Further experimentation showed that we could also make flat films of gel inside which the chloroplasts were entrapped; these proved to be much better than the spheres.

The films tended to be rather fragile, and broke up if stirred or shaken for more than 5 min; this problem was overcome by covering a solid grid support with the sodium alginate-chloroplast mixture and dropping calcium chloride on to this mixture with a pipette. The result is that the support becomes embedded in the calcium alginate, giving a flat, rigid structure. The principle is similar to that of reinforced concrete. Muslin was initially used as the strengthening grid but we have also tried nylon and stainless steel. Stainless steel has, in fact, proved to be the best so far, giving strong, easy-to-make films which do not fragment on shaking.

The photosynthetic activity of immobilized chloroplasts was assayed in an oxygen electrode and compared to that of free chloroplasts, using both potassium ferricyanide (oxygen evolution) and methyl viologen (oxygen uptake) as electron acceptors, as shown in Table 1. It was necessary to use only small amounts of chloroplasts for assay because the alginate film was such that it lined the inside of the electrode cavity and the buffer solutions flowed past it. As the illumination of the film was from one side only, that part of the film nearest the light shaded the rest of the film; this was considerable when 100 µg total chlorophyll was immobilized in the film. Stirring would have overcome this problem with free chloroplasts, but this was not possible with immobilized ones. Therefore, it was decided to decrease the amount of chlorophyll present so that the illumination should be saturating over the entire area of the film. Table 1 clearly shows

Table 1 Photosynthetic electron transport activity of immobilized and non-immobilized chloroplasts

	Free chloroplasts	Immobilized chloroplasts Spheres	Films
O ₂ evolution rate (µmol O ₂ evolved per mg chlorophyll per h)	200	98	198
O ₂ uptake rate (µmol taken up per mg chlorophyll per h)	207	107	205

Chloroplasts were isolated essentially as in ref. 13 and resuspended in a solution of 10 mM NaCl, 10 mM HEPES, pH 7.5 (2 mg chlorophyll ml⁻¹). Assays were carried out in a Rank oxygen electrode at 25 °C. The reaction mixture contained, in a final volume of 4 ml, chloroplasts containing 25 µg of chlorophyll, 50 mM HEPES, pH 7.5, and 10 mM NaCl. For O₂ evolution, the electron acceptor was potassium ferricyanide (3 mM). For O₂ uptake, the electron acceptor was methyl viologen (50 µM)¹⁴. Both O₂ evolution and uptake were measured in the presence of 2 µM carbonyl cyanide *p*-trifluoromethoxyphenol hydrazone, uncoupling agent. Alginate spheres were made by mixing chloroplasts containing 25 µg chlorophyll with 1 ml of a solution of 1% sodium alginate + 50 mM HEPES (pH 7.5) + 1% bovine serum albumin (BSA) and dropping this mixture into 100 ml of 0.1 M CaCl₂ + 50 mM HEPES (pH 7.5). The films were made by mixing chloroplasts containing 50 µg chlorophyll with 1% sodium alginate + 50 mM HEPES (pH 7.5) to final volume of 300 µl. A nylon grid, 1 × 2 cm, mesh 45, aperture size 0.32 mm, was covered in 150 µl of this mixture, and 3 ml of 0.1 M CaCl₂ + 50 mM HEPES (pH 7.5) were dropped onto the film with a pipette.

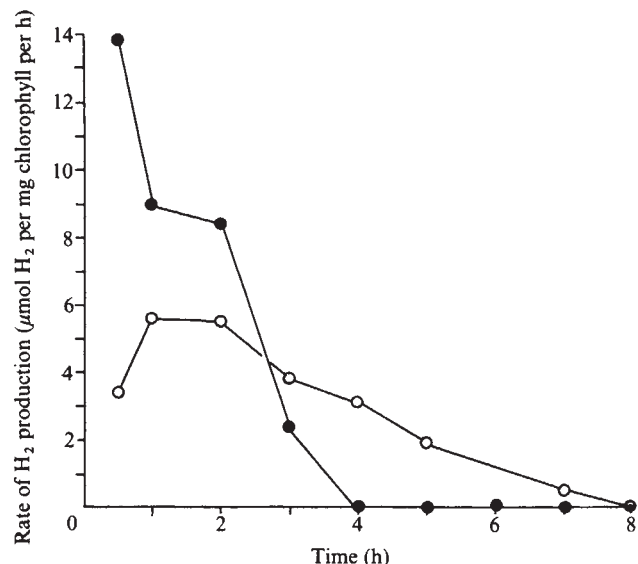


Fig. 1 Rate of hydrogen production against time for system (3)—platinum-polyvinyl alcohol and methyl viologen with spinach chloroplasts. Reaction conditions were as shown for system (3) in Table 2 legend. (Data are from a longevity experiment separate to those shown in Table 2.) ●, No components immobilized; ○, chloroplasts, electron carrier and H₂-evolving catalyst in alginate gel film. Oxygen trap present in both.

that the photosynthetic electron transport activity of the film-immobilized chloroplasts was not diminished by the immobilization process compared to that of free chloroplasts. However, the spheres only showed 49% of the activity of free chloroplasts.

Next, we were interested to see if immobilized chloroplasts could be used to produce hydrogen gas, as can free chloroplasts^{1,2} using hydrogen-producing catalysts and the appropriate electron carriers. Three catalytic systems were used: (1) *Clostridium pasteurianum* hydrogenase with *Spirulina maxima* ferredoxin as the electron carrier; (2) *Desulfovibrio desulfuricans* (strain Norway) hydrogenase with methyl viologen as electron carrier, and (3) a synthetic catalyst platinum-polyvinyl alcohol¹⁰ with methyl viologen as carrier. The films were made by mixing double the required amount of chloroplasts, electron carrier and catalyst to a final volume of 150 µl. This was then mixed with 150 µl of a solution containing 4% sodium alginate (BDH batch 6097490), 1% bovine serum albumin and 50 mM HEPES, pH 7.5, giving a final sodium alginate concentration of 2%. A stainless steel grid (1 × 2 cm, 20 mesh, aperture size 0.99 mm) was covered with 150 µl of the final mixture and then 3 ml of solution containing 0.1 M CaCl₂ and 50 mM HEPES, pH 7.5, was dropped on to the film from an automatic pipette.

Table 2 shows that hydrogen-evolution was observed using the immobilized chloroplasts, with quite good rates in immobilized systems (1) and (3). However, the percentage of the same reaction system where none of the components are immobilized (figures in parentheses), shows that the rate retained by system (1) after immobilization is 24%, but that for system (3) is 67%. In an immobilized system we have never retained more than 25% of the maximum hydrogen-evolving activity attainable (that is system (1), non-immobilized).

At this stage, the important point is not the absolute rates of hydrogen production (as compared to free chloroplasts) but that the immobilized chloroplasts will evolve hydrogen at reasonable rates. Following work showing the generation of photovoltages and photocurrents⁴, it was shown that immobilized chloroplasts and algal cells will produce photocurrents in a three-electrode system when they are coated on an electrode and a suitable potential applied¹¹; however, these systems do not produce hydrogen. We have attempted to develop a catalytic solar energy converter which uses water as the source of electrons and protons, and which ultimately gives rise to hydrogen. The system described here is the only one known which fulfils these criteria.

Table 2 Rates of hydrogen production from immobilized and non-immobilized systems

	H ₂ evolved (μmol per mg chlorophyll per h)		
	System (1): chloroplasts + <i>C. pasteurianum</i> hydrogenase + <i>S. maxima</i> ferredoxin	System (2): chloroplasts + <i>D. desulfuricans</i> hydrogenase + methyl viologen	System (3): chloroplasts + Pt.PVA + methyl viologen
No components immobilized	21.0 (100%)	7.1 (100%)	7.2 (100%)
Chloroplasts, electron carrier and H ₂ -evolving catalyst in alginate gel film	5.0 (24%)	1.1 (15%)	4.8 (67%)
No components immobilized, oxygen trap omitted	10.7 (51%)	2.8 (39%)	0 (0%)
Chloroplasts, electron carrier and H ₂ -evolving catalyst in alginate film, oxygen trap omitted	1.8 (9%)	0.2 (3%)	0 (0%)

Chloroplasts (type C)¹⁵ were prepared essentially as described in ref. 13 and finally suspended in 10 mM NaCl, 10 mM HEPES, pH 7.5 (7.5 mg chlorophyll ml⁻¹). Assays for H₂ evolution were carried out in 15-ml glass vials. The reaction mixture (final vol 2 ml in 50 mM HEPES, pH 7.5) contained 100 μg chlorophyll, 1% BSA (fraction V powder). When present, the oxygen trap consisted of 100 μmol of glucose, 10 units glucose oxidase, 1,000 units catalase and 20 μl ethanol. System (1) *C. pasteurianum* hydrogenase added to excess; *S. maxima* ferredoxin (25 nmol per vial). System (2) *D. desulfuricans* (strain Norway) hydrogenase, added to excess; methyl viologen was present at 50 μM. System (3) Pt. PVA = platinum-polyvinyl alcohol, containing the equivalent of 125 μg platinum per vial, methyl viologen 50 μM. Hydrogen evolution was assayed over 2 h at 11,000 l and 25 °C using a gas chromatograph as described in ref. 16. Alginate gels were made as described in the text.

The *in vitro* production of hydrogen from reduced substrates, for example EDTA and ascorbate, using dyes as mediators is well established, and efficiencies of 5% conversion of monochromatic light have been reported^{4,12}. However, catalytic water-splitting systems have only been substantiated thus far using chloroplast membranes, where the lack of sustained and linear hydrogen production rates over many hours has not allowed the meaningful measurement of solar energy conversion efficiencies.

For the development of a large-scale solar converter, we are investigating the replacement of as many biological components as possible with synthetic compounds, largely because of the instability and expense of the former. System (3) of Table 2 is of particular interest in this respect because in it we have replaced the ferredoxin and hydrogenase used in system (1) with methyl viologen and platinum-polyvinyl alcohol, respectively. In Fig. 1 we show that if calcium alginate-immobilized chloroplasts are used with the two synthetic catalysts of system (3), although the initial rates are lower than for free chloroplasts, we increase the longevity of the system twofold. Preliminary work shows that the inorganic catalysts are not well retained in the gel after initial entrapment, as some leakage occurs. There is, however, no leakage at all of the chloroplasts themselves. Separate immobilization of the synthetic and biological catalysts, and their subsequent incorporation into the alginate gels, supported on a rigid matrix, is the next phase of our work.

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Note added in proof: Initial experiments with free chloroplasts, in collaboration with Dr A. Harriman, have given quantum efficiencies for hydrogen-producing systems (1), (2) and (3) of 0.126 (12.6%), 0.025 (2.5%) and 0.032 (3.2%), respectively (see Table 2).

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Secondary isotope effects in studies using radiolabelled folate tracers

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The use of isotopically labelled compounds as metabolic tracers in *in vivo* and *in vitro* studies is particularly important in folate biochemistry, where the natural coenzyme forms occur in transient and trace amounts. However, during our studies on the whole body metabolism of radioactive folate tracers, we have now observed that some forms of isotopically labelled folate are biologically distinguishable from the unlabelled carrier molecules by an as yet unidentified secondary isotope effect. Foliates excreted in the urine of rats dosed previously with mixtures of ¹⁴C- and ³H-labelled folate derivatives are apparently ³H enriched, that is contain more ³H than ¹⁴C relative to the dosed compound. This apparent ³H enrichment was largely due to the enhanced absorption of ³H-folate from the intestine, confirmed by studies with everted sac preparations of rat jejunum in which it was found that the ³H-labelled folate in the mixture was transported from the mucosal into the serosal compartment at a faster rate than both the ¹⁴C-labelled folate and the unlabelled carrier. Secondary isotope effects were also observed on ion-exchange chromatography, ³H-labelled folates with ³H at the C-9 position eluting fractionally earlier than the corresponding unlabelled or [2-¹⁴C]folate from DEAE-cellulose, a behaviour similar to that reported earlier for isotopically labelled 2-aminopurine¹ and several amino acids². Such isotope effects may be more widespread and more important than is generally accepted.

Folic acid (Fig. 1) is commercially available (Radiochemical Centre) in three radioactively labelled forms: labelled with ¹⁴C (at the C-2 position) or with ³H, either nominally (at the 3', 5', 7, 9 positions, with 33.2% at C-9) or generally (at the 7, 9 positions only, with 41% at C-9). Extensive catabolism of the folate molecule occurs *in vivo*³ and therefore, to facilitate metabolite identification and quantification, we routinely use mixtures of ¹⁴C- and ³H-labelled folate tracers in our whole animal studies.

Urine collected 0–48 h after the administration of mixtures of [2-¹⁴C]- and [3', 5', 7, 9-³H]folate (10–100 μg per kg body weight) to rats contains a large excess of ³H over ¹⁴C whereas faecal samples contain more ¹⁴C than ³H compared with the dose⁴. Chromatographic analysis on DEAE-cellulose and Sephadex G15 revealed that the catabolites present in the urine that were labelled with ³H only (³H₂O, *p*-acetamidobenzoate

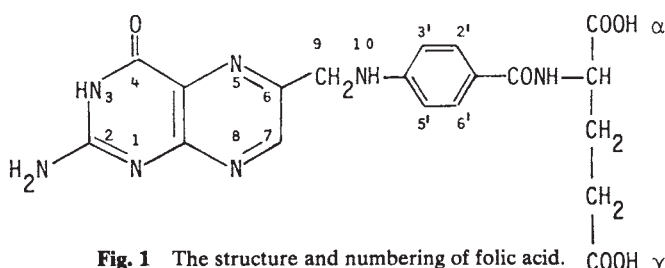


Fig. 1 The structure and numbering of folic acid.

and *p*-acetamidobenzoyl-L-glutamate) accounted for only 50% of the ^3H excess, the residue being due to an enhanced ^3H to ^{14}C ratio (compared with the dosed compound) of the intact folates excreted (largely 5-methyl-5,6,7,8-tetrahydrofolic acid, 10-formyl-5,6,7,8-tetrahydrofolic acid and unmetabolized tracer). The evidence indicated that either ^3H -folate was being absorbed faster or that $[2\text{-}^{14}\text{C}]$ folate was being absorbed more slowly from the intestine with respect to unlabelled folate. Everted sac preparations of rat jejunum⁵ were incubated for 30 min with radiolabelled 10-formyl folic acid, folic acid or pterin-6-COOH and the serosal fluid examined by HPLC. For each of the intact folates examined, an increased serosal ^3H to ^{14}C ratio was found compared with the starting material (Table 1). The ^{14}C specific radioactivity of the folate in the mucosal and serosal compartments remained constant. As enhanced ^3H -folate absorption was even more pronounced when mixtures of $[2\text{-}^{14}\text{C}]$ - and $[7,9\text{-}^3\text{H}]$ folate were used compared with mixtures of $[2\text{-}^{14}\text{C}]$ - and $[3',5',7,9\text{-}^3\text{H}]$ folate, the effect was due to the ^3H atoms at the 7 and 9 positions. Because the ^3H to ^{14}C ratio remained constant in the serosal and mucosal compartments when mixtures of $[2\text{-}^{14}\text{C}]$ - and $[7\text{-}^3\text{H}]$ pterin-6-COOH (synthesized from a mixture of $[2\text{-}^{14}\text{C}]$ - and $[7,9\text{-}^3\text{H}]$ folic acid⁶) were used, the important site seems to be position 9, although this assumes that the pterin-6-COOH is transported by a similar mechanism to that of folate.

The isotope effect is not confined to biological situations. When mixtures of $[2\text{-}^{14}\text{C}]$ - and ^3H -folate are eluted from DEAE-cellulose columns, the ^3H to ^{14}C ratio decreases across the peak, that is, ^3H -labelled molecules elute slightly faster than the ^{14}C -labelled molecules. Similar separations of mixtures of isotopically labelled compounds have been reported previously. We analysed our chromatographic data by the method of Klein *et al.*⁷ using the Probit transform to provide the values M (the volume at which 50% of the compound has eluted), ΔM (the difference between the value of M for the unlabelled and labelled compound) and $\Delta M\%$ $[(\Delta M/M) \times 100]$ (Table 2). Graphical plots of the Probit transform of the cumulative percentage of the compound eluted against elution volume gave

Table 1 The relative amounts of ^3H -labelled, ^{14}C -labelled and unlabelled pteridines transported into the serosal compartment by everted sac preparations of rat jejunum

Compound and isotope mixture	$^3\text{H}:^{14}\text{C}$ ratio		^{14}C :absorbance ratio	
	Starting compound	Serosal compound	Starting compound	Serosal compound
$[2\text{-}^{14}\text{C}]$ folic acid, $[3',5',7,9\text{-}^3\text{H}]$ folic acid and unlabelled folic acid	1	1.25	1	1
$[2\text{-}^{14}\text{C}]$ folic acid, $[7,9\text{-}^3\text{H}]$ folic acid and unlabelled folic acid	1	1.29	1	1
$[2\text{-}^{14}\text{C}]$ 10-formyl folic acid, $[3',5',7,9\text{-}^3\text{H}]$ 10-formyl folic acid and unlabelled 10-formyl folic acid	1	1.10	1	1
$[2\text{-}^{14}\text{C}]$ pterin-6-COOH, $[7\text{-}^3\text{H}]$ pterin-6-COOH and unlabelled pterin-6-COOH	1	0.98	1	1

Everted rat jejunal sacs were incubated with 1 mM solutions (containing 0.1 μCi each isotope) of the appropriate mixture for 30 min. The combined serosal fluid (from three sacs) was then applied to a Whatman Pastisil 10 SAX Magnum 9 HPLC column (50 cm $\times$ 0.94 cm) and eluted with 0.10 M potassium phosphate buffer, pH 5.0, at 600–800 p.s.i. to resolve transported unmetabolized starting material from any contaminant metabolites. The isotope content was determined by scintillation counting and unlabelled compound from its UV absorbance at 254 nm. The $^3\text{H}:^{14}\text{C}$ ratios in the derivatives found in the tissue at the end of each experiment were similar to those of the serosal compartment.

Table 2 The effect of isotope substitution on elution from DEAE-cellulose ion exchange columns

	ΔM (ml)	$\Delta M\%$
$[2\text{-}^{14}\text{C}]$ folic acid	No separation detected	
$[3',5',7,9\text{-}^3\text{H}]$ folic acid	-4.30	1.59
$[7,9\text{-}^3\text{H}]$ folic acid	-5.10	2.02
$[2\text{-}^{14}\text{C}]$ 10-CHOFA	No separation detected	
$[3',5',7,9\text{-}^3\text{H}]$ 10-CHOFA	-0.45	0.26
$[2\text{-}^{14}\text{C}]$ 5-MeTHF*	No separation detected	
$[3',5',7,9\text{-}^3\text{H}]$ 5-MeTHF*	-1.25	0.47
$[2\text{-}^{14}\text{C}]$ pterin-6-COOH	No separation detected	
$[7\text{-}^3\text{H}]$ pterin-6-COOH	No separation detected	

Mixtures of ^{14}C - and ^3H -labelled compounds (0.1 μCi each isotope) and unlabelled carrier (5 mg) were applied to DEAE-cellulose columns (1.5 cm $\times$ 30 cm) and eluted with a 0–1.2 M NaCl gradient in 0.05 M sodium phosphate buffer, pH 7.0. The ^{14}C , ^3H and UV absorbance of each fraction (5 ml) was determined and the degree of isotope fractionation calculated. Values of ΔM are quoted with respect to the elution position (M) of the unlabelled carriers; in the calculation of $\Delta M\%$ the sign is artificially reversed to indicate the displacement of labelled from unlabelled compound in the conventional manner. The approximate elution positions of the carriers are: folic acid, 265 ml; 10-CHOFA (10-formyl folic acid), 175 ml; 5-MeTHF (5-methyl-5,6,7,8-tetrahydrofolic acid), 199 ml; pterin-6-COOH, 208 ml.

* Isolated from rat urine after oral administration of ^{14}C - and $[3',5',7,9\text{-}^3\text{H}]$ folic acid.

a series of parallel lines for the unlabelled and corresponding radioactively labelled compounds, demonstrating their homogeneity and absence of co-chromatographing impurities. For all folates examined, the ^{14}C -tracer and the authentic unlabelled compound (measured from its UV absorbance) were well matched, with ΔM values close to zero, whereas the ^3H -tracer eluted before both of them (Table 2). We have also observed similar though less marked mismatches on Sephadex G-15 chromatography (data not shown). The chromatographic data confirm the conclusion reached from the everted sac studies that ^3H incorporation at the C-9 position is the important factor in the aetiology of the isotope effect, as $\Delta M\%$ for $[7,9\text{-}^3\text{H}]$ folic acid was greater than for $[3',5',7,9\text{-}^3\text{H}]$ folic acid and was negligible for $[7\text{-}^3\text{H}]$ pterin-6-COOH in similar conditions (Table 2). The role of the C-9 ^3H is also emphasized by the decrease in the size of the isotope effects on formylation at the N-10 position.

The C-9 ^3H -folate derivatives behaved as if they carried an effective net reduced charge, as they eluted early from anion-exchange columns at neutral pH. Several mechanisms have been proposed to explain isotope effects of this type. Gottschling and Freese¹ suggested that an unspecified electronic effect, resulting in a change in the pK value of the amino group, was responsible for the chromatographic separation of ^3H -labelled 2-aminopurine from unlabelled 2-aminopurine. More recently, Klein and his co-workers² have provided evidence that isotope-induced changes in hydrogen bonding and local solvent structure may be important. Similar conclusions have been reported⁸ for isotopic separations of a variety of deuterated and unlabelled hydrocarbons by hydrophobic HPLC where differences in C–H and C–D bond perturbation by solvent was considered crucial. Isotope-related changes in solvent/bonding effects would obviously be important in folate–protein interactions, either directly, if, for example, the C-9 position was involved in bonding, or indirectly, by contributing to an effective overall decrease in the net charge of the folate molecule. Because incorporation of a ^3H atom at the C-9 position leads to a pronounced effect on the transport of folate across the intestine, careful screening for secondary isotope effects is essential in enzymatic and metabolic studies involving such tracers.

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MATTERS ARISING

Ordering of FeNi in clear taenite from meteorites

CLEAR taenite in meteorites¹ contains 48–57% Ni and is optically anisotropic², unlike taenite (γ Fe,Ni) which is isotropic. We have proposed² that clear taenite is composed of ordered FeNi, a phase first identified in iron meteorites by Albertsen *et al.*³. Mehta *et al.*⁴ have obtained additional evidence for ordering in clear taenite from their electron-optical studies of a large grain, which one of us (R.S.C.) discovered in the Estherville meteorite. However, they interpret their data to indicate that clear taenite is composed of regions of ordered FeNi, which are 1–3 μ m in diameter, in a matrix of disordered γ (taenite) of similar composition. We argue here that this conclusion is in all probability incorrect.

On ordering of FeNi, cubic taenite inverts to the tetragonal CuAu or L1₀ structure. Because of this symmetry change, three sets of intergrown crystallites of ordered FeNi, which are oriented in mutually perpendicular directions, form from a single crystal of taenite. These crystallites, which can be observed in reflected light under crossed polars², are typically 1–10 μ m in diameter.

Mehta *et al.*⁴ observed extra superlattice diffraction spots from the L1₀ structure in preferentially thinned regions of clear taenite in which the [110] direction was parallel to the electron beam. The thicker regions which did not show extra diffraction spots were interpreted by Mehta *et al.* to be disordered γ . We suggest instead that the thicker regions actually contained sets of ordered crystallites with [101] and [011] directions parallel to the beam. On the original taenite axes, the unit cell of ordered FeNi is C-centered and has dimensions which are very close to those of taenite³. Ordered crystallites with [101] and [011] parallel to the beam would therefore show diffraction patterns that are indistinguishable from those of disordered γ . Thus we are confident that Mehta *et al.* misidentified ordered FeNi crystallites as disordered taenite. If we are correct, all regions of {100} sections of clear taenite will show extra superlattice spots, as in this orientation all three sets of crystallites show superlattice diffraction spots.

Although they acknowledge that our interpretation is consistent with their data, Mehta *et al.*⁴ strongly emphasize their own conclusion that clear taenite contains ordered FeNi regions in a disordered γ matrix. Other contrary evidence is provided by Mössbauer^{3,5} and optical² studies of meteoritic metal, which have failed to detect disordered γ of the size, compo-

sition and abundance that Mehta *et al.* propose. We still conclude^{2,6} that clear taenite is a new mineral, ordered FeNi.

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Differences in fluidity between bilayer halves of plasma cell membranes

ASYMMETRY in the composition of membrane bilayer halves raises the possibility that the individual monolayers have independent physical properties. Recently, Schroeder¹ proposed a method for analysing the fluidity of the individual monolayers of tumour cell plasma membranes. He used an impermeable reagent, trinitrobenzenesulphonic acid (TNBS), covalently linked to outer monolayer amino groups, to quench the fluorescence of *trans*-parinaric acid, a probe which distributes in both monolayers. He contends that the fluorescence of the outer monolayer was quenched and that the elevated polarization of the residual fluorescence reflected a lowered mobility of the *trans*-parinaric acid located in the inner monolayer.

We suggest that TNBS linked to outer monolayer phospholipid amino groups quenches the fluorescence of *trans*-parinaric acid in the two monolayers to a different extent. Because any energy transfer process reduces the donor probe fluorescence lifetime², the observed elevation of the polarization of the unquen-

ched *trans*-parinaric acid fluorescence may reflect the impact of the transfer process on fluorescence lifetime³ rather than probe mobility. Our arguments are based on the following considerations.

(1) The width of membrane lipid bilayers (between opposite polar head-groups) is generally agreed to be less than 50 Å (ref. 4). (2) *trans*-Parinaric acid, 9, 11, 13, 15, all *trans*-octadecatetraenoic acid is an extended molecule, orientated with its carboxyl group near the polar headgroup. We expect the relatively inflexible tail of *trans*-parinaric acid to be near the middle of the bilayer with the midpoint of its transition dipole (between C-12 and C-13) ~6 Å to either side of the centre of the bilayer. (3) R_0 , the distance at which energy transfer between parinaric acid and covalently linked TNBS is 50%, is calculated by standard procedures⁵, applicable to membrane bilayer energy transfer to be 23 Å. (4) For a bilayer 48 Å wide, *trans*-parinaric acid chromophores in the outer and inner monolayers will be ~18 Å (0.78 R_0) and 30 Å (1.3 R_0), respectively, from the trinitrophenyl chromophores. (5) With the geometry specified above, we calculate by the method of Wolber and Hudson⁶ that, to achieve the 44% reduction of fluorescence of *trans*-parinaric acid by energy transfer between *trans*-parinaric acid and TNBS reported by Schroeder, a TNBS surface density of 0.6 TNBS/ R_0^2 is required. The calculations show that ~60% of the outer monolayer and ~27% of the inner monolayer fluorescence are transferred. (6) Fluorescence lifetimes are reduced by energy transfer to an extent similar to the detected change in fluorescence quantum yield^{2,5}. For isotropically rotating probes, the Weber–Perrin equation⁷ predicts that the lifetime changes in *trans*-parinaric acid caused by the presence of TNBS are alone sufficient to account for the observed elevation of the polarization.

As it seems that the motion of linear probes like *trans*-parinaric acid in membranes is not isotropic^{8,9}, the extent to which the observed steady-state polarization depends on the probe fluorescence lifetime is a function of both the rate of chromophore motion and its angular extent³. These parameters are probably influenced by membrane proteins and cholesterol¹⁰ and can only be extracted by nanosecond polarization measurements³. We have, however, observed that the steady-state polarization of parinaric acid probes, confined to the surface monolayer of a serum lipoprotein and quenched by surface-bound energy transfer acceptors, is elevated to an extent comparable to that reported by Schroeder in tumour cell membrane bilayers (L.A.S., unpublished results).

We believe that a general validation of Schroeder's method requires absolute proof that only outer monolayer *trans*-parinaric acid is quenched and quenched completely by energy transfer to TNBS. We believe that Schroeder can validate his conclusions about tumour cell bilayers by measuring r_{∞} , the limiting value of the anisotropy at long times following excitation. Because r_{∞} is independent of the fluorescence lifetime and because the residual fluorescence in the presence of TNBS labelling probably arises predominantly from the inner monolayer, an elevated r_{∞} in the presence of TNBS would provide good evidence for the difference in the mobility of inner and outer monolayer *trans*-parinaric acid probes.

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SCHROEDER AND KINDEN REPLY—We thank Sklar and Doody for their comments and appreciate their concern. It is regrettable that these authors treat a complex biological membrane system, the LM cell plasma membrane, as if it were a model membrane. No experimentally determined values for LM cell membranes were presented. In contrast, we have continued our work in this area and substantiated the use of trinitrophenyl (TNP) groups as fluorescence quenchers and asymmetry determination agents in several systems^{1–9}. The erroneous assumptions made in the above critique are as follows.

First, comments (1)–(4) of Sklar and Doody assume the width of the LM cell membrane to be less than 50 Å. In contrast, from micrographs obtained with an RCA EMU-36 electron microscope, we have determined the width of the LM

cell plasma membrane to be ~80 Å. Thus, half the width of the membrane would be 40 Å, not 23 Å as proposed by Sklar and Doody. The additional width of the LM cell membrane seems to be due to the presence of proteins. This is especially important because at least 10 times more TNP groups were attached to protein amino substituents than to phospholipids. The fluorophore of *trans*-parinaric acid is about 15 Å from the carboxyl end, similar to the distance of the fluorescent anthroyl group in anthroyl-stearate from its carboxyl end^{10,11}. Therefore, quenching across the bilayer would be expected to be less than 0.004 times as efficient as in the outer monolayer. Studies of model systems indicate that there is little energy transfer from one leaflet to the acceptor in the opposite one if R_0 is much less than 40 Å (ref. 12).

Table 1 Fluorescence decay of *trans*-parinarate in LM cell plasma membranes

Cell or membrane treatment	$t_{1/2}$ (ns)	
Untreated membranes	5.9	8.9
Membranes + 100 μ M TNP-glycine	5.8	9.1
Pretreatment of whole cells with 4 mM TNBS to trinitrophenylate outer monolayer amino groups ¹	6.0	9.1

$t_{1/2}$ Represents the halftime for decay for each decay component. Plasma membrane vesicles were resuspended in phosphate-buffered saline (100 μ g ml⁻¹) and *trans*-parinarate was incorporated as described previously¹. Trinitrophenylated membranes were prepared also as stated previously¹.

Second, our reported increases in polarization were not due to altered fluorescence lifetime. Evidence obtained with an Ortec 9200 nanosecond spectrometer (courtesy of Dr Lynwood Yarbrough, University of Kansas School of Medicine) showed that the fluorescence lifetime of *trans*-parinarate was not altered. Two decay components in unquenched membranes were observed as shown in Table 1. Addition of 100 μ M TNP-glycine to plasma membrane vesicles (100 μ g protein per ml phosphate-buffered saline, 24 °C, pH 7.4) maximally quenched fluorescence but did not alter the halftimes of decay. TNP-glycine has been used as a non-penetrating quencher in very low-density lipoprotein to quench completely the fluorescence of *trans*-parinarate^{3–5}. As shown in Table 1, trinitrophenylated membranes also did not show altered halftimes for *trans*-parinarate fluorescence decay. In all cases of maximal quenching by TNP-glycine or TNP groups attached to the membrane, the fluorescence decay curves were superimposable. In addition, similar data were obtained using a second fluorescence

probe molecule, 1,6-diphenyl-1,3,5-hexatriene². Lastly, *trans*-parinarate was incorporated into lipid vesicles made from plasma membrane lipid extracts. The quenching agent TNP-glycine showed no differences in polarization between the inner and outer monolayer. This would be expected because lipid vesicles would have a randomized symmetric distribution of lipid molecules across the bilayer.

Thus, our data in no way support the general photophysical artefact alluded to by Sklar and Doody. Indeed, if the quenching data are artefactual, all three probe molecules (*trans*-parinarate, 1,6-diphenyl-1,3,5-hexatriene and *N*-phenyl-1-naphthylamine) and membranes from cells supplemented with choline, *N,N'*-dimethylethanolamine, *N*-monomethylethanolamine and ethanolamine should have given essentially the same data—increased fluorescence polarization in the presence of quenching agent. This was not the case². *N*-phenyl-1-naphthylamine did not show differences in polarization in the presence of quenching agents. *trans*-Parinarate incorporated into plasma membranes from *N,N'*-dimethylethanolamine and *N*-monomethylethanolamine-supplemented cells also did not show differences in polarization in the presence of quenching agents.

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BOOK REVIEWS

Higher education: retrospect and prospect

Fred Dainton

IN ONE of his many memorable prose passages Sir Peter Medawar has reminded us that, if a science is really advancing, more and more of the facts are correlated by an intellectual framework comprising "general statements of steadily increasing explanatory power and compass" which relieve us of the burden of remembering every fact — the corollary being that books conveying the essence of the subject should progressively decrease in size. By this token the subject of higher education is either not a science or is retrogressing, for what has been written in the past two decades — and there is much — is often lengthy and devoid of generally accepted unifying ideas.

The largest effort in this field, dinosaurian in size (if not in ultimate fate), is that masterminded by the Carnegie Commission on Higher Education, which has resulted in 21 reports and 64 sponsored studies on the average larger than either of the two books reviewed here. Collectively, and in massive detail, the Carnegie publications take a close look at higher education in the USA, with the aim of recommending a way forward which might suffice to carry 'the system' to the end of this millennium.

These two books are as unlike the Carnegie series as could be. They are short, innocent of graphs, unbuttressed by statistics, and the prose of each is simple, direct, unambiguous and pleasingly free from the polysyllabic jargon of the pundits of education and sociology. Both are written by professional economists, and both are essentially personal statements. But there the resemblance ends.

Lord Robbins's book, as befits the elder statesman and Chairman of the Committee

Higher Education Revisited. By Lord Robbins. Pp.117. (Macmillan: London/Holmes and Meier: New York, 1980.) £12, \$34. *Higher Education for the Future.* By Charles Carter. Pp.160. (Basil Blackwell: 1980.) £7.50. Available from Biblio Distribution Centre, Totowa, NJ, \$15.

on Higher Education which sat for three years at the beginning of the 1960s — the report of which is undoubtedly the single most important and influential document on higher education ever to be issued in the United Kingdom — is an urbane contemplation of those recommendations which were accepted in 1963 in the light of what has happened since in British universities. Sir Charles, the founding Vice-Chancellor of the University of Lancaster and 20 years Robbins's junior, tells us in pithy prose what the whole British 'system' (if that is not too strong a word) of post-sixth-form education is, what he would like it to become and what he thinks may well happen to it by the time he is Lord Robbins's age.

Inevitably, some topics are common to the two books. For instance, both touch on student unrest and show their deep revulsion for the methods and aims of the extremists amongst the student militants of the late 1960s and early 1970s which they see as a denial of the academic values of truthfulness and rationality. I would go further than either and assert that the single most important factor in the diminution in public esteem of the universities was the unacceptable behaviour of that tiny minority of militant students which was given wholly disproportionately magnified coverage by the media. This made it much easier for successive governments to restrict the resources they were prepared to make available to universities.

Robbins poses a severe test for any university subject — "Can it be taught so as to enlarge the general powers of the mind?" I don't think I have ever heard this criterion discussed, let alone applied, at any meeting concerned with science curricula at departmental or faculty level, and I feel that some lecturers and textbook authors are so didactic that their influence is mind-constricting rather than expanding. Many of us will draw some comfort from Robbins's statement of "the desirability of working on something that does not at once arouse spontaneous enjoyment". Scientists should also read, mark and inwardly digest what he says about specialization in undergraduate courses. He acknowledges that advances in arts and

science come predominantly from specialists but points out that success for the majority of graduates in the western world rests on their broad educational foundations and their versatility, and he recalls the often forgotten qualification to the expansion explicit in the principle of access to higher education by all who are "able and willing" that there should be many more broader courses.

As a Scot, he frequently looks disdainfully southward at the system in England and Wales which forces irreversible specialization on schoolchildren who are far too immature and inexperienced to make wise choices. For 12 years I have been convinced that a broad span of studies in the sixth form is overwhelmingly advantageous even if it necessitates either a longer academic year or more four-year undergraduate courses. A glorious opportunity to do both now presents itself as the size of the 18-year-old age group declines, but I'll wager it will not be seized. Other reforms advocated by Robbins which would help towards this end are a break of at least a year between school and university and, to contain costs, a system of undergraduate student loans, repayment of which would be required only if the annual income exceeded some stated value, an eminently sane and equitable suggestion which would be easy to administer. However, I most strongly disagree with the notion of loans for postgraduates. Were this to become a reality and Research Council and DES studentships to disappear, much valuable research and trained manpower, especially in the sciences and engineering, would be lost, to the detriment of the country as a whole.



Lord Robbins



Sir Charles Carter

Robbins has much to say that is wise and thoughtful on the value of research, on its relation to and effects (good and bad) on teaching and on the desirable size of institutions — always a matter of subjective judgement rather than logical argument. But I was surprised that he considers universal tutorial teaching to be generally impracticable. Why ever not? Given that the staff/student ratio is 1/9, this means a tutorial load of only 9 hours per week on a one-to-one basis. Surely this or the 4½ hours per week on a one-to-two basis are not excessive additions to the other duties of academics. Including laboratory demonstrating, I did a 22 contact-hour week in Cambridge in 1949 and was none the worse for it.

Lord Robbins's reflections on internal institutional government, on relations between universities and the state (who am I to dispute his high regard for the University Grants Committee?), on the justification of the twin donnish privileges of tenure and freedom to choose his own work, on why more does not mean and has not meant worse, on the binary system and on many other matters, are full of wisdom and experience. Moreover the book has the peculiar charm that each chapter is cast in the form of a letter to a fictitious correspondent who, one surmises, is highly sceptical of the value of higher education. I am reminded of the dictum of Lord Robbins's fellow Scot, Robert Louis Stevenson, that "Every book is, in an intimate sense, a circular letter to the friends of him who wrote it". The reader cannot fail to respond to the warmth and human kindness which lie behind the adoption of this epistolary device and the manifest benignity of the author, factors which — such is the nature of the human psyche — are more persuasive than the statistical data which Lord Robbins freely admits to be absent.

Sir Charles Carter's approach is entirely different. He epitomizes the rational man and is the natural enemy of cant. His prose is precise, crisp and, in some cases, sharp. His arguments command respect for their cogency even if the premises are sometimes mere assertions. He is prepared to question every assumption, overt or covert, on which he considers the British higher education system to be based — there are no sacred cows for him. Whereas Robbins commands attention by charming the reader, Carter, by deflating many of his readers' preconceptions and ascribing qualities of bravery and foolishness to those who attract his approbation or otherwise, may irritate and repel. I would urge those whose initial reaction takes this form to put aside the irritation and to persist, because this is a stimulating book deserving a wide readership.

He starts with a lucid account of the system as it is. Though as faithful to the subject as a photograph, there are illuminating comments, for example, about "social responsiveness" (words much used

in the launching of the polytechnics) as really meaning that "public sector colleges are more easily ordered about" (with which I and, I suspect, many US State College and University Presidents would agree) and about how the UGC allocates resources (with which I do not agree). He then delineates the "needs and desires" of society and of individuals, pointing out that society really needs sufficient people with a range of skills able to learn and to adapt quickly, who can be competent citizens, manage change and yet preserve "culture" — defined as "what is worth remembering" — and handing on as a legacy to successive generations. After upbraiding Robbins for "a certain sloppiness of thinking" in assigning a "right" to higher education for all the "able and willing", his chapter on the needs and desires of individuals states that intellectual development is not a sufficient individual need and should be balanced by ethical, social and emotional development. That sentiment is, of course, in praise of virtue and against sin but is imprecise and does not lead to any practical conclusion other than the rather lame one that entry tests should not be restricted to cognitive skill. The next chapter raises the question of the wastefulness and inefficiency of higher education, and the need for better planning. Yet, after a scornful look at the Oakes Committee Report as being weak in its recommendations, he seems to reject planning and to welcome overlap of functions between different types of institution on the ground that planners are "insensitive to the need for variety" and are hostile to new ideas, especially if they did not conceive them — no names, no pack drill!

At this point Sir Charles turns to the future, AD 2000, dealing in turn with the sort of institutions we need, the curriculum, research, and the Government as the (inevitable) paymaster. He gives a high priority to Two-Year Community Colleges (an echo of the USA?) and, believing that scholarship is an essential activity for a good teacher whereas research is not, he therefore wants a differentiation of institutions in higher education according to their research roles, with more inter-institutional mobility for students. The longest chapter deals with the undergraduate curriculum, the inertia of which clearly appals him. He sees the essential elements as a specialist core, related subjects, optional courses (which seem to be directed towards making better citizens) and "the examination of some implications of the specialist studies", though the possible nature of these "implications" is, regrettably in my view, left as an exercise for the reader. He writes much common sense on teaching methods and shares my often-repeated view on the invalid reification into a 'subject' of an area of knowledge divided from the rest for the convenience of the division of labour. Examinations are regarded as necessary

but he sees much room for improvement in diagnosing the strengths and weaknesses of candidates.

The word 'research', which he regards as a portmanteau containing many different meanings, next comes under his critical scrutiny. He regards it as being of little value in relation to teaching and as a narrowing activity for many, involving little opportunity for originality and independent critical thought. We all have our views on these matters, but he is surely right when he argues for removing the contractual burden 'to research' from university teachers and for a more positive research policy both nationally (which would carry with it the institutional differentiation mentioned above) and within institutions. But he says nothing about the criteria which such policies should satisfy.

To achieve these reforms Sir Charles proposes new machinery including a Higher Education Grants Committee maintaining contact with institutions through a regionally based inspectorate (*sic!*), a single governing body instead of the usual academic senate and predominantly lay council. The members of the governing body would be chosen more for competence than their representativeness. Twenty-five per cent of the revenue of institutions should come from fees in order to remind everyone that costs should be adjusted to output, though this would in fact provide an unhealthy incentive to maintain student numbers irrespective of quality and reduce the influence of the Grants Committee as a regulatory instrument. He also wants loans for students at least for the third and later years. Finally, after a brief encounter with Illich, a conclusion that technology will not cut the ground from under traditional institutions (which, however, ought to teach for 40 weeks per year rather than 30) and the advocacy of a *right* of access to holders of two GCE A-Level passes, Sir Charles proffers us views of what would happen in the next 20 years.

One wonders why this last chapter was written, for I could find nothing new in it other than a *cri de coeur* for more people "to ask awkward questions" and a means for them to do so. But, if this is the necessary condition for change, and if one cannot invent people to do this or if the present system fails to produce them, does that mean we must endure the inertia he so deplores? I do not believe so, for I have more faith in the institutions and the individuals within them to change, but both need the stimulus of books like these. □

Sir Fred Dainton is a chemist who was Vice-Chancellor of Nottingham University from 1965 to 1970 and Chairman of the University Grants Committee from 1973 to 1978. He is currently Chairman of the National Radiological Protection Board and the British Library Board.

Beyond Tansley's 'ecosystem'

Peter D. Moore

Grasslands, Systems Analysis and Man. Edited by A. I. Breymer and G. M. Van Dyne. Pp. 950. (Cambridge University Press: 1980.) £65, \$140.

It is now 45 years since A. G. Tansley coined the term 'ecosystem'. The concept of looking at nature as an integrated whole, with intricate connections between its various component parts, both living and non-living, has now been exploited to an extent which is no doubt far beyond Tansley's wildest dream. The limitations of purely mental models have now been cast aside with the advent of sophisticated computer analogues and precise mathematical analysis. Consequently, the work of the International Biological Programme (IBP) has been designed, carried out and assembled using a systems approach to the ecology of various habitats.

The book reviewed here is a further addition to the various syntheses of IBP data which are currently being published and it is concerned with grassland ecosystems. It differs from the previous volume, *Grassland Ecosystems of the World* edited by R. T. Coupland (reviewed in *Nature* 283, 317; 1980), in that it is concerned with seeking a description of common processes within grasslands rather than with surveying the variations within the world's grassland types. It is thus a book in which the systems analysis approach to ecosystem study is fully developed and illustrated. Since it has involved 38 different authors from all over the world, it must have been a headache to edit in order to maintain some uniformity of style and approach.

The book is arranged in three parts. In the first, the total system is analysed into its components — abiotic, autotrophic, small and large herbivore, invertebrate and vertebrate predator, and decomposer subsystems respectively. In the second part, the ecosystem is assembled and such total system processes as nutrient cycling, energy flow and trophic structure are considered. Finally, in Part III, the use of the systems approach in the management and exploitation of grassland ecosystems is discussed.

The general approach of editors and authors has been to begin with a simple, easily conceived, low-resolution model of the various systems and subsystems covered and to develop it and elaborate it as necessary. One danger with this is that the initial model can become oversimplified to the extent that it is no longer helpful. This is the case, for example, in the general ecosystem model presented by Breymer in the Introduction, in which energy and matter

are not separated and both appear to cycle. Hinds and Van Dyne, however, use the same technique very effectively when describing the abiotic subsystem of the grassland ecosystem. Taking water movements, they construct a simple, low-resolution model involving only three components and then expand it to illustrate the complexity of subcomponents within these major divisions and, even more important, the intricacy of likely paths of water movement within such a high-resolution model.

All chapters have strongly developed review components and each is equipped with its own bibliography. Thus, for example, the chapter on the autotrophic subsystem by Singh *et al.* reviews the various models which have been developed to describe the photosynthetic process and its relationship to environmental factors. Particular attention is given to the Waggoner, Chartier and Lommen models of carbon uptake by the leaf.

Among the herbivores, it is evident that there is considerably more information concerning the large herbivores than the small (invertebrates). For the former, a great deal of interesting information is collated, relating not only to energy flow within the large herbivore, but also to

food selectivity on the part of many such grazers.

Ecologists concerned with the practicalities of grassland management, rather than the satisfying symmetry of computer-drawn curves, will also find something to interest them in this book. Data are presented from experimental grazing trials in the USA which examine such subjects as the influence of stock density upon floristic composition.

Overall, the book leaves two lasting impressions. One is the value of the systems analysis approach to understanding processes in the ecosystem. The second is the advantages gained by an international approach to the development of such models for habitats with similar structure and life-form composition. In this way one can proceed beyond the high-resolution, complex model to general statements concerning the function of such an ecosystem and the nature of the global variation upon the general theme. This volume, with its predecessor, Vol. 18 in the series, represents a significant step towards this goal. □

Peter D. Moore is Senior Lecturer in the Department of Plant Sciences at King's College, University of London.

Reviews of galactic research

C. Pritchett

Photometry, Kinematics and Dynamics of Galaxies. Edited by D.S. Evans. Pp. 492. (Department of Astronomy, University of Texas: 1980.) \$16 + postage.

THIS volume reports on a conference that was held in Austin, Texas, in August 1979. The proceedings of the conference communicate the current excitement over 'nearby' galaxies — a field of research (once considered prosaic by many) that has been transformed by a new generation of technology.

Many of the major scientific results that were presented in Austin are in press, or else have already been published. This of course detracts from the usefulness of the conference proceedings, but nevertheless reflects favourably on the scientific importance of the meeting to participants and their co-workers. Of particular interest are papers (by T.X. Thuan and W. Romanishin, and by R.E. Schild and T.C. Weekes) that purport to show that so-called 'cD' galaxies in poor clusters are simply giant ellipticals. Other exciting new results include the detection of a 'thick-disk' component in SO galaxies (by D. Burstein), and the detection of rotation in the bulges of spiral galaxies (by J. Kormendy and G. Illingworth). This latter

finding is in contrast to the (now well-known) non-rotation of elliptical galaxies — a result that is considered at length in this volume. Other topics of extensive discussion include the kinematics of HI and HII in spiral galaxies, and the surface photometry of spirals.

Perhaps the most commendable feature of this volume is the presence of several excellent review papers — by S.E. and K.M. Strom (on the effects of environment on the evolution of elliptical and disk galaxies), by K.C. Freeman (on disk galaxies), by M. Capaccioli (on the kinematics of early-type galaxies) and by J. Kormendy (on component analysis of galaxy luminosity profiles). Two other reviews, on galaxy photometry (by G. de Vaucouleurs and by H. Corwin), provide a historical perspective which may well be instructive to younger astronomers. Though somewhat tersely written, the papers in this compendium maintain an up-to-date and authoritative focus on areas of current interest; all will be of some value to specialists and browsers alike.

Photometry, Dynamics and Kinematics of Galaxies can be recommended to all readers with an interest in modern extragalactic astronomy. The editor, D.S. Evans, and contributors are to be commended for making this volume available sufficiently promptly so as to ensure its immediate scientific value. □

C. Pritchett is a Research Associate at Dominion Astrophysical Observatory, Victoria, Canada.

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The evolution of coastal East Anglia

Brian Funnell

The Pre-glacial Pleistocene of the Norfolk and Suffolk Coasts. By R.G. West. Pp. 300. (Cambridge University Press: 1980.) £40, \$84.

ANYONE who has looked down an East Anglian estuary, or across the East Anglian tidal flats and marshes of the present day, will readily appreciate the variety of environments represented there. Assume these environments to have been destroyed and re-created three or four times, under alternately cold and temperate climates, at almost identical topographic levels, and you have some idea of the complexity of the deposits which record the history of the pre-glacial coastlands of East Anglia.

The first comprehensive attempt to interpret these deposits was made by Clement Reid, an officer of the Geological Survey of England and Wales, towards the end of the nineteenth century. For half-a-century afterwards little significant further work was carried out on them until P. Thomson applied modern pollen analytical techniques to the West Runton Freshwater Bed. S. L. Duigan's doctoral thesis of 1954 confirmed Thomson's conclusion that the peat bed at West Runton represented an almost entire interglacial cycle, and at the same time revealed the difficulties that would be encountered by anyone attempting a full interpretation of the whole sequence of Cromer Forest Bed deposits. In 1958 Dr, now Professor, R. G. West started work on a full pollen analytical and environmental interpretation of the Cromer Forest Bed Series and his marathon studies are enshrined in this comprehensive monograph.

The book records for posterity, with faithful care, all the problems, difficulties and complexities of the Cromer Forest Bed deposits as they have revealed themselves to one persistent observer over a period of years. Although it is accompanied by a thorough commentary and separate discussion of some general aspects, such as sea-level changes and environmental interpretation, there are no over-slick summaries that would enable a casual reader to raid the book for quick answers on vertebrate or plant community evolution, stratigraphical correlation or sea-level changes. Each piece of evidence is carefully weighed, considered and recorded, but never forced into a simple framework for the sake of easy conclusions. It thus stands as a record of work that is both complete and still in progress.

Most readers would be well advised to arm themselves with a liberal supply of bookmarks to facilitate cross-reference between the text and the numerous tables,

plates and figures (the latter conveniently provided as a separate booklet in a back pocket to the book), as these need to be continuously referred to as the text is read. It is indeed one of the attractions of this book that it is not just a presentation of the author's conclusions but also a complete do-it-yourself kit that will enable readers to reassess and reinterpret the basic data which have been so assiduously collated.

Overall Professor West concludes that the Cromer Forest Bed Formation has accumulated during four main pollen stages. The story starts with an account of the last marine transgression of the Norwich Crag Formation, which extended over the Chalk of north-east Norfolk during the initial substage 'a' of the cool Pre-Pastonian. (There is some reference to the earlier pre-glacial marine deposits of the Ludhamian, Thurnian, Antian and Baventian stages in the text, but only to complete the description of a particular section or borehole or to round off comparisons of floras from later stages.)

The Cromer Forest Bed proper begins with the main Pre-Pastonian (sub-stages 'b' to 'd'), which is represented by freshwater and fluvial deposits containing cool floras occurring at between -3 and +5m OD. It is followed by the Pastonian stage, containing temperate pollen, sometimes starting and ending with freshwater deposition, but including a substantial coastal marine phase, at levels between -9 and +8m OD. The succeeding Beestonian stage is again cold, with evidence of frozen-ground conditions, but is also in part coastal-marine in origin. Its deposits occur at between +2 and +8m OD in a limited area in north Norfolk. Finally the Cromerian stage *sensu stricto* is temperate, substantially freshwater, but with a significant mid-Cromerian marine or estuarine phase, and occurs at levels of between -7 and +8m OD over a wide area. The sequence concludes with early Anglian arctic peaty muds which are sealed in immediately under the first true glacial deposits on the East Anglian coast.

Correlation of the East Anglian stages with comparable stages identified in the contemporary deposits of the Netherlands is still uncertain, probably owing to the close similarity of successive temperate floras at this period in the Quaternary and to the strong influence of local environments on pollen spectra on both sides of the North Sea at that time. The absence of a definitive correlation does not however detract from the intrinsic interest of the detailed ecological and geographical changes which occurred along the East Anglian coast before the arrival of glaciers.

Professor West is to be congratulated on a major work of scholarship, which certainly matches the contribution of Reid and will undoubtedly become a classic in its own right. □

Brian Funnell is Professor of Environmental Sciences at the University of East Anglia.

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